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TITLE: The Role of the EGF Receptor and Vitamins A and D in Development and Progression of Breast Cancer to More Malignant Hormones Independent Phenotypes

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13. ABSTRACT *(Maximum 200*

Epidermal growth factor receptor (EGFR) is a strong prognostic indicator for poor survival rate independent of estrogen receptor status, suggesting that EGFR overexpression is an important step in the progression to estrogen independence. We have shown repression of EGFR mRNA and protein by 1,25-dihydroxyvitamin D_3 in MCF7, T47D, and BT549 breast cancer cells. Functional mapping of the EGFR promoter in these cells has revealed a DNA segment between -536 and -478 that resembles a consensus vitamin D response element (VDRE) and confers a vitamin D response upon both the homologous and a minimal heterologous promoter. *In vitro* footprinting and gel shift assays have confirmed the binding of the vitamin D receptor (VDR) and an unknown partner to this putative VDRE. An sp1 binding site was also identified in close proximity and shown to bind sp1 from nuclear extract only when the VDRE is unoccupied. Mutational analysis and functional studies using a minimal heterologous promoter provide evidence that the VDR in concert with its unknown partner mediates EGFR repression through displacement of sp1. In BT549 cells these experiments also suggest that there are additional cell specific factors that allow the VDR to mediate repression of EGFR through low affinity VDRE half-sites at higher vitamin D concentrations. A model is proposed that accounts for these events at the molecular level.

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THE ROLE OF THE EGF RECEPTOR AND VITAMINS A AND D IN THE DEVELOPMENT AND PROGRESSION OF BREAST CANCER TO MORE MALIGNANT HORMONE-INDEPENDENT PHENOTYPES

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THE ROLE OF THE EGF RECEPTOR AND VITAMINS A AND D IN THE DEVELOPMENT AND PROGRESSION OF BREAST CANCER TO MORE MALIGNANT HORMONE-INDEPENDENT PHENOTYPES

INTRODUCTION

Despite the fact that approximately 60% of human breast cancers contain estrogen receptor at the time of diagnosis, only about two thirds of these tumors will respond to anti-estrogen therapy, and many of those which do respond initially will develop resistence to anti-estrogen therapy over time. This progression to a more malignant form of the disease is often associated with an increase in substances known as growth factors or growth factor receptors that provide the tumor with an alternate signal telling the cells to grow. One such growth factor receptor whose presence in breast tumors strongly correlates with poor clinical prognosis and the lack of response to anti-estrogen therapy is the epidermal growth factor receptor (EGFR). This overall goal of this project is to understand how vitamins A and D control the level of this growth factor receptor that is functionally implicated in the development and progression of breast cancer.

BODY

Research Accomplishments (see attached manuscript for details)

Our previous results suggested that vitamin D and retinoid compounds may partially mediate their biologic effect on growth in breast cancer cells by the differential modulation of oncogene products and growth factor receptors such as EGFR. In support of this, we had demonstrated that those breast cancer cell lines with lower levels of EGFR expression, specifically MCF7 and T47D, had the greatest amount of growth inhibition when treated with 1μM of 1,25-dihydroxyvitamin D_3, analog C (a 1,25-dihydroxyvitamin D_3 analog with chemical name 1α,25-$(OH)_2$-16-en-23-yn-26,27-F_6-vitamin D_3), 9-cis RA, or all-trans RA, while those cell lines with higher levels of EGFR expression, such as BT474 and BT549, responded less significantly, or not at all. We observed that downregulation of EGFR expression after vitamin D and retinoid treatment correlated with growth inhibition only for the MCF7 and T47D cell lines. While growth inhibition was observed in BT474 cells, they unexpectedly showed EGFR upregulation. Further, BT549 cells showed no significant growth inhibition in the face of significant EGFR down-regulation. These discordant results suggested that the growth inhibitory and EGFR downregulatory effects of the vitamin D and retinoid compounds are cell specific and not necessarily dependent phenomenon. In order to more fully understand EGFR up- or downregulation in the context of the growth inhibition induced by the retinoid and vitamin D compounds, we felt it first necessary to establish the molecular basis of EGFR regulation by the vitamin D, and subsequently, retinoid compounds in different breast cancer cells. We have previously demonstrated that 840 nucleotides of the EGFR promoter in the context of a reporter gene are able to mediate inductive and repressive responses to vitamin D that mimick the differential regulation of endogenous EGFR expression in BT474 and BT549 cells, respectively, suggesting the involvement of cell specific factors in the vitamin D controlled regulation of growth and EGFR expression in breast cancer cells.

We have now characterized the factors and sequences that are important for EGFR downregulation in MCF7, T47D, and BT549 breast cancer cells in response to vitamin D treatment.

Starting with the previously identified region of the EGFR promoter which localized the vitamin D responsive region to within 840 nucleotides, we mapped the primary vitamin D response to a region of the promoter between nucleotide positions -536 and -478 that contains a putative VDRE and transcription factor sp1 binding site. Using functional reporter assays and DNA-protein binding studies, we have determined that the EGFR promoter does in fact contain a functional VDRE that spans nucleotides 531 to 516 upstream of the translation start site. This VDRE, with a sequence of GGGTCCACAGGGGCA (half sites underlined), demonstrates remarkable similarity to the classical PuG(G/T)TCA DR3 consensus VDRE.

Initial identification of this VDRE was facilitated by transient transfection of a CAT reporter construct containing 840 nucleotides of the EGFR promoter into MCF7, T47D, and BT549 cell lines followed by 1μM vitamin D treatment. All three cell lines demonstrated vitamin D repression of promoter activity, and progressive deletions of the EGFR promoter allowed for localization of the response to a region between the SacII and Bsu36I restriction sites at nucleotide positions -536 and -478, respectively. In the BT549 cell line, elimination of this 58 nucleotide region of the EGFR promoter did not completely negate the 1μM vitamin D response as it did in MCF7 and T47D cells, but rather only reduced the magnitude of the repression by a factor of 2. This suggested that the SacII to Bsu36I sequence contributes to the overall 1μM vitamin D effect in BT549 cells, but may not exclusively mediate it. Only upon additional deletion of promoter sequence to 145 bases upstream of the translation start site was a complete loss of a 1μM vitamin D effect observed, suggesting the existence of a second vitamin D responsive region of the EGFR promoter between nucleotide positions -478 and -145 that functions in BT549 cells.

The identification of a common vitamin D responsive region of the EGFR promoter between nucleotides -536 and-478 in MCF7, T47D, and BT549 cell lines lent support to our hypothesis that vitamin D repression of EGFR expression is mediated through a putative VDRE located within this region of the EGFR promoter. In mapping this element, an unexpected result was obtained with the identification of the second 1μM vitamin D responsive region of the EGFR promoter between nucleotides -478 and -145 in BT549 cells only. Consequently, we hypothesized that this second region may mediate a 1μM vitamin D response through cell specific factors that allow utilization of multiple low affinity VDR binding sites located within it. Indeed, when the sequence of this second 1μM vitamin D responsive region of the EGFR promoter is examined, many PuGGTCA motifs are apparent.

Further characterization of the vitamin D responsive region of the EGFR promoter spanning the SacII to Bsu36I sites by *in vitro* footprinting using nuclear extracts from MCF7, T47D, and BT549 cells demonstrated defined regions of protein binding whose sequences were hypothesized to be sp1 and VDR half site binding motifs. While EMSAs with purified sp1 and VDR showed simultaneous binding of theses factors, the use of nuclear extracts demonstrated the specific but mutually exclusive binding of sp1 and the VDR with an unknown partner. Mutations introduced into the putative sp1 and VDR binding sites confirmed the specificity of binding, while transfection of wild type and mutant sequences in the context of a heterologous, minimal promoter driven CAT construct demonstrated the functionality of the VDRE. In T47D cells at 1μM vitamin D, and in BT549 cells at 0.1μM vitamin D, mutation of either the VDRE or the sp1 binding site completely abolished the vitamin D effect. However, at 1 μM vitamin D, mutation of the VDRE and sp1

binding site were both required in order to completely abolish the vitamin D response in BT549 cells, suggesting once again the involvement of BT549 cell specific factors and possibly other lower affinity VDR binding sites within the SacII to Bsu36I sequence that gain functionality upon a higher concentration of vitamin D.

One way of interpreting our finding of a second vitamin D responsive region of the EGFR promoter between nucleotides -478 and -145, which only represses in BT549 cells, and only at a vitamin D concentration of 1μM, might involve binding of the VDR to multiple, low affinity VDRE half-sites within this region that interferes with the binding of TFIIB to other members of the transcription initiation complex. This is especially probable in light of the fact that the EGFR promoter has numerous transcriptional start sites and VDRE half sites within this region, the latter of which by themselves have been shown bind the VDR and mediate gene transactivation in other systems. The fact that this region of the EGFR promoter only responds at a high vitamin D concentration supports this mechanism of vitamin D gene repression through low affinity VDREs, while its observation only in BT549 cells, and not MCF7 and T47D cells, suggests the involvement of one or more factors specific to BT549 cells. It should be noted that EMSAs were performed using BT549 nuclear extract and restriction fragments spanning the region of the EGFR promoter between positions -478 to -145. In each case, extract failed to show binding of a complex having immunoreactivity to the VDR. However, when incubated with excess purified VDR and RXRß proteins (i.e., >50 fold excess when compared to the amount needed to see binding to the SacII to Bsu36I restriction fragment), complexes were noted with several of the DNA probes containing VDRE-like half-sites. These results further support our speculation that BT549 cell specific factors coordinate the binding of the VDR and other necessary factors to lower affinity VDRE half sites within the -478 to -145 region of the EGFR promoter, and that such binding contributes to the vitamin D repressive effect on EGFR expression at a concentration of 1μM.

The binding of the VDR to vitamin D responsive DNA sequences has mostly been associated with a corresponding activation of gene expression. These "positive" VDREs are comprised of direct repeats of the sequence PuGGTCA or GGTTCA separated by three nucleotides. Recently, it has been suggested that vitamin D is able to mediate repressive responses by binding to "negative" VDREs which differ from the traditional DR3 by a few nucleotides. Such differences in the base composition of the VDRE is believed to cause distinctive conformational changes in the VDR and its transactivation domain that result in transcriptional repression rather than activation. The functional VDRE identified by us differs from classical upregulatory VDREs by one nucleotide in its 5' half site (underlined): GGGTCC. It has been demonstrated in other reports that the last nucleotide in the 5' VDRE half site tends to be an adenine and is found in up to 93% of VDREs that bind the VDR and activate transcription. This suggests that such a difference is not of minimal importance.

Additionally, transcriptional repression mediated through the VDRE identified in the EGFR promoter appears to involve functional interference by the VDR of the activity of positive transcription factors that bind to the EGFR promoter, and in particular, positive transcription factor sp1. We have demonstrated through EMSA the mutually exclusive binding of either sp1 or VDR and an unknown partner to the vitamin D responsive region of the EGFR promoter, suggesting that VDR competes with sp1 for binding and therein exerts transcriptional repression. This is supported

by our functional data in T47D cells treated with 1μM vitamin D and in BT549 cells treated with 0.1μM vitamin D, showing that mutation of either the VDRE or sp1 sites, which results in loss of corresponding nuclear factor binding as assessed by EMSA, causes a complete loss of a vitamin D response. This strongly suggests that to exert its repressive effect in T47D cells and in BT549 cells at 0.1μM vitamin D, the VDR must first displace bound sp1 and that mere binding of VDR and its heterodimeric partner to the EGFR VDRE is not sufficient. An alternative hypothesis to the displacement of sp1 by the VDR is that sp3, a factor related to sp1 but shown to inhibit its function by competitive binding, binds to this region of the EGFR promoter and in some fashion acts with the VDR to downregulate transcription. This idea lacks support, however, as an antibody directed against sp3 failed to recognize it as one of the nuclear binding factors in complex with the SacII-Bsu36I restriction fragment in EMSAs even though it was found to be present in nuclear extract by western analysis.

Our functional data also supports the assertion that the identified VDRE, by nature of its nucleotide sequence, mediates negative rather than positive transcriptional responses by vitamin D. That is, mutation of the sp1 site, which abolishes sp1 but not VDR binding, does not subsequently allow the VDR to mediate a positive (rather than negative) transcriptional response as it would be predicted to do if the sequence were a classical "positive" VDRE. Instead, it simply negates or reduces the vitamin D effect. So as to rule out the unlikely possibility that vitamin D simply cannot mediate positive transcriptional responses in MCF7, T47D, and BT549 cells for some unknown reason, we transfected a SV40 promoter driven CAT construct containing an upstream osteocalcin VDRE into each of these cell lines and have observed an induction of CAT in response to vitamin D treatment in each case. Since the nature of the base substitutions introduced by us to mutate the VDRE were drastic and abolished VDR binding, it may be interesting to assess whether or not more conservative changes in the VDRE nucleotide sequence that maintain VDR binding would result in a vitamin D mediated up- instead of downregulation of EGFR, thus confirming or refuting the functional significance of the identified half-site mismatches in our VDRE.

While our results clearly indicate that transcriptional repression mediated through our identified EGFR VDRE involves the disruption of sp1 from its proximal binding site, additional functional data in BT549 cells indicates that the VDR is also able to mediate a degree of transcriptional repression in addition to that which is caused by sp1 displacement, at least in this cell line. At a vitamin D concentration of 1μM in BT549 cells, loss of sp1 binding still allows for a repressive, but blunted, vitamin D response in the context of an intact VDRE and vice versa. We speculate that this level of repression seen in the face of lost sp1 binding might involve one or more additional BT549 cell specific factors that operate through protein-protein contacts to disrupt the activation properties of the initiation complex. An explanation for the maintenance of a repressive, but blunted, vitamin D response with mutation of the VDRE might involve the binding of the VDR and/or its partner to other lower affinity VDRE half sites located within the SacII to Bsu36I restriction fragment, or alternatively may involve protein-protein interactions mediated by cell specific factors.

In terms of VDR's binding partner, it is known that the RXRs, and under some circumstances, the RARs can partner and bind with the VDR to DNA sequences. In our studies we have noted that the VDR binds to our identified VDRE with a nuclear protein that shows no

immunoreactivity to antibodies raised against RXRα, RXRß, RXRγ, RARα, RARß, and RARγ. Sequences which bind the VDR:RXR heterodimer with the highest affinity tend to be positive VDREs (which have an A rather than a C as the last nucleotide in their 5' half site), suggesting that transcriptional repression mediated by vitamin D may not involve heterodimerization of the VDR with RXR. It is interesting to note that while we were able to demonstrate binding of a VDR:RXR heterodimer to our VDRE using purified proteins, a parallel experiment performed with crude nuclear extract demonstrated the formation of a different VDR complex. Therefore we postulate that the VDRE identified in this study gains specific repressive, rather than inductive, functionality through a unique sequence that favors VDR binding as a heterodimer with an as of yet unidentified nuclear factor.

The binding properties of the nuclear factor postulated to dimerize with the VDR and bind to our VDRE were explored in titration experiments. While it is interesting that an excess of purified VDR and sp1 proteins can result in the loss of the nuclear complex containing the VDR and its unknown partner, it is even more intriguing to note the corresponding appearance of a slower migrating complex containing both sp1 and VDR proteins. This sp1/VDR complex was also seen with EMSA using purified factors only, and was found to be disruptable with the addition of increasing amounts of nuclear extract. Given these results, we have postulated a model that attempts to explain the molecular details surrounding vitamin D mediated repression of EGFR expression in MCF7, T47D, and BT549 breast cancer cells. We propose that the repression pathway is initiated through ligand activation of the VDR, followed by subsequent dimerization with an unknown partner and binding to the negative VDRE spanning nucleotides -531 to -516 of the EGFR promoter. Presumably through a combination of unproductive transcriptional conformational changes in the VDR's transactivation domain brought about by this heterodimerization and/or binding to a "negative" VDRE, displacement of transcription factor sp1 from its proximal binding site then occurs and results in repression by disruption of functional sp1 interactions with the rest of the transcription machinery. Such a disruption may be mediated by the VDR and its partner directly, or through additional factors that coordinate protein-protein contacts. Additionally, in BT549 cells we propose that there is also the direct interaction of the VDR with one or more cell specific factors that allows for recognition and activation of VDRE half sites within a second vitamin D responsive region of the EGFR promoter.

Training

Kenneth McGaffin completed and successfully defended his doctoral dissertation, and resumed the clinical portion of his MD/PhD training. However, he continues to return to the lab to work on this project as his schedule allows. He will be granted both the MD and the PhD degree concurrently at the end of his medical training. James Welch has replaced Ken McGaffin on this project and is receiving training in the areas of gene regulation and breast cancer while preparing his thesis proposal.

APPENDICES

KEY RESEARCH ACCOMPLISHMENTS

- A DNA segment between -536 and -478 of the EGFR promoter that resembles a consensus vitamin D response element (VDRE), confers a vitamin D response upon both the homologous and a minimal heterologous promoter in MCF7, T47D, and BT549 breast cancer cells

- The vitamin D receptor (VDR) and an unknown partner bind to this putative VDRE, and an sp1 binding site was also identified in close proximity and shown to bind sp1 from nuclear extract only when the VDRE is unoccupied

- VDR in concert with its unknown partner mediates EGFR repression through displacement of sp1

- In BT549 cells, there are additional cell specific factors that allow the VDR to mediate repression of EGFR through low affinity VDRE half-sites at higher vitamin D concentrations

REPORTABLE OUTCOMES

- Manuscript: McGaffin, K.R. and Chrysogelos, S.A. Identification and characterization of a response element in the EGFR promoter that mediates transcriptional repression by 1,23-dihydroxyvitamin D_3 in breast cancer cells. Manuscript submitted to Molecular Endocrinology.

- McGaffin, K.R. and Chrysogelos, S.A. Identification and characterization of a response element in the epidermal growth factor receptor promoter that mediates transcriptional repression by 1,25-dihydroxyvitamin D_3 in breast cancer cells. The Era of Hope: The department of defense Breast Cancer Research Program Meeting, Washington, DC, October 31-November 4, 1997.

MANUSCRIPTS AND ABSTRACTS ATTACHED

- Manuscript: McGaffin, K.R. and Chrysogelos, S.A. Identification and characterization of a response element in the EGFR promoter that mediates transcriptional repression by 1,23-dihydroxyvitamin D_3 in breast cancer cells. Manuscript submitted to Molecular Endocrinology.

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IDENTIFICATION AND CHARACTERIZATION OF A RESPONSE ELEMENT IN THE EPIDERMAL GROWTH FACTOR RECEPTOR PROMOTER THAT MEDIATES TRANSCRIPTIONAL REPRESSION BY 1,25-DIHYDROXYVITAMIN D_3 IN BREAST CANCER CELLS

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Epidermal growth factor receptor (EGFR) is a prognostic indicator and potentially a causal factor in the development and progression of breast cancer. This study was designed to examine the molecular details surrounding the mechanism of action of vitamin D in breast cancer cells both in terms of effect on EGFR expression and antiproliferative properties. The growth properties of 1,25-dihydroxyvitamin D_3 (vitamin D) and a vitamin D analog (analog C) were examined in MCF-7, T47D, BT474 and BT549 breast cancer cell lines. Significant growth inhibition was observed in MCF-7, T47D and BT474 cells by 8 days of treatment with either compound at a concentration of 1μM, while BT549 cells showed no significant growth inhibition. Three days treatment with either compound at 1μM resulted in a 40-70% reduction in EGFR mRNA in MCF-7, T47D, and BT549 cells as measured by RNase protection assay, while a 300-600% increase was observed in BT474 cells. Western blotting using membrane fractions from BT474 and BT549 cells demonstrated that EGFR protein levels correlated with these changes in mRNA levels. These data imply that growth inhibition and EGFR down-regulation by vitamin D compounds may be linked in some, but not all, breast cancer cells.

To better understand the regulation of EGFR by vitamin D compounds in breast cancer cells, we felt it was important to first establish the molecular basis of this regulation. Transient transfection in breast cancer cells of a reporter construct containing 840bp of the EGFR proximal promoter demonstrated that the EGFR promoter could mediate vitamin D-induced

Keywords: **Epidermal Growth Factor Receptor, Gene Expression, Vitamin D, Transcriptional Repression, Vitamin D Responsive Elements**

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-96-1-6295.

changes in transcriptional activity that parallel the changes observed in endogenous EGFR mRNA and protein. Sequence analysis and functional mapping of the EGFR promoter revealed a DNA segment between nucleotides -536 and -478 that resembles a consensus vitamin D response element (VDRE) and confers a vitamin D response upon both the homologous and a minimal heterologous promoter in MCF-7, T47D, and BT549 cells. *In vitro* footprinting and gel shift assays confirmed the binding of the vitamin D receptor (VDR) to the VDRE as a heterodimer with an unknown partner that was not a member of the RXR or RAR families. An Sp1 binding site was also identified in close proximity to the VDRE, and it was found that while purified Sp1 and VDR could bind simultaneously, Sp1 could not bind when the VDRE was occupied by the VDR and its unknown partner. Mutational analysis and functional studies using the homologous and a minimal heterologous promoter provided evidence that in breast cancer cells the VDR in concert with its unknown partner mediates EGFR repression through the displacement of Sp1. In BT549 cells these experiments also suggested that there are additional cell specific factors that allow the VDR to mediate repression of EGFR through low affinity VDRE half-sites at higher vitamin D concentrations. Titration studies with purified VDR and Sp1 and nuclear extract from breast cancer cells indicated the presence of one or more nuclear factors in breast cancer cells that in concert with the VDR mediate the displacement of Sp1. Based on these findings, a model has been developed that attempts to explain the molecular details of EGFR repression by vitamin D compounds in breast cancer cells.

Identification and Characterization of a Response Element in the EGFR Promoter that Mediates Transcriptional Repression by 1,25-Dihydroxyvitamin D_3 in Breast Cancer Cells

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Running Title: Characterization of a VDRE in the EGFR Promoter

Keywords: vitamin D, EGF receptor, vitamin D responsive element, gene regulation, breast cancer

ABSTRACT

Repression of EGFR mRNA and protein by 1,25-dihydroxyvitamin D_3 has been documented in MCF7, T47D, and BT549 breast cancer cells. Functional mapping of the EGFR promoter in these cells has revealed a DNA segment between -536 and -478 that resembles a consensus vitamin D response element (VDRE) and confers a vitamin D response upon both the homologous and a minimal heterologous promoter. *In vitro* footprinting and gel shift assays have confirmed the binding of the vitamin D receptor (VDR) and an unknown partner to this putative VDRE. An sp1 binding site was also identified in close proximity and shown to bind sp1 from nuclear extract only when the VDRE is unoccupied. Mutational analysis and functional studies using a minimal heterologous promoter provide evidence that the VDR in concert with its unknown partner mediates EGFR repression through displacement of sp1. In BT549 cells these experiments also suggest that there are additional cell specific factors that allow the VDR to mediate repression of EGFR through low affinity VDRE half-sites at higher vitamin D concentrations. A model is presented which attempts to characterize these events at the molecular level.

INTRODUCTION

Overexpression of epidermal growth factor receptor (EGFR) occurs in a majority of breast cancers (1,2) and has been repeatedly correlated with more malignant or advanced disease (3,4), poor prognosis (5-7), and/or likely patient failure on endocrine therapy with the appearance of hormone independent growth (8-10). Binding of ligand (such as EGF or TGF-α) mediates a mitogenic effect on many cell lines, including breast cancer cell lines, through activation of EGFR., with the 170 kd transmembrane glycoprotein receptor serving as an upstream effector in a variety of signal transduction pathways via its tyrosine kinase activity (11-14). EGF is able to stimulate DNA replication and cell division in both normal and malignant cells (15-17) in part by countering the expression of a differentiated phenotype (18).

1,25-dihydroxyvitamin D_3, all-trans retinoic acid (RA), and 9-cis retinoic acid (RA) are compounds that have been shown to limit proliferation of and promote differentiation in breast cancer cells (19-23). Additionally, vitamin D and retinoid compounds have been shown to serve as effective antiproliferative agents in breast cancer when used in conjunction with antiestrogens (24-27), or in the case of hormone independent growth, as promising alternatives to traditional chemotherapy (28-30). In earlier studies (31) we suggested that the vitamin D and retinoid compounds may partially mediate their biologic effect on growth in breast cancer cells by the differential modulation of oncogene products and growth factor receptors such as EGFR. In support of this, we have demonstrated that those breast cancer cell lines with lower levels of EGFR expression, specifically MCF7 and T47D, had the greatest amount of growth inhibition when treated with 1μM of 1,25-dihydroxyvitamin D_3, analog C (a 1,25-dihydroxyvitamin D_3 analog with chemical name 1α,25-$(OH)_2$-16-en-23-yn-26,27-F_6-vitamin D_3), 9-cis RA, or all-trans RA, while those cell lines with higher levels of EGFR expression, such as BT474 and BT549, responded less

significantly, or not at all. We observed that downregulation of EGFR expression after vitamin D and retinoid treatment correlated with growth inhibition only for the MCF7 and T47D cell lines. While growth inhibition was observed in BT474 cells, they unexpectedly showed EGFR upregulation. Further, BT549 cells showed no significant growth inhibition in the face of significant EGFR down-regulation. These discordant results suggested that the growth inhibitory and EGFR downregulatory effects of the vitamin D and retinoid compounds are cell specific and not necessarily dependent phenomenon. In order to more fully understand EGFR up- or downregulation in the context of the growth inhibition induced by the retinoid and vitamin D compounds, we felt it first necessary to establish the molecular basis of EGFR regulation by the vitamin D, and subsequently, retinoid compounds in different breast cancer cells. To this end, we have demonstrated that 840 nucleotides of the EGFR promoter in the context of a reporter gene are able to mediate inductive and repressive responses to vitamin D that mimick the differential regulation of endogenous EGFR expression in BT474 and BT549 cells, respectively (31). This suggests the involvement of cell specific factors in the vitamin D controlled regulation of growth and EGFR expression in breast cancer cells.

1,25-dihydroxyvitamin D_3 mediated gene expression is generally accepted to be a transcriptional event (32). Vitamin D exerts its effects by the binding to and activation of nuclear VDRs (33). The VDR belongs to the class II family of nuclear receptors which also includes the RXRs (34). Upon homo- or heterodimerization, the VDR binds to specific nucleotide sequences or response elements (REs) within the regulatory regions of genes and exerts an inductive or repressive effect on transcription (32,33), depending upon its binding partner and the sequence context of the RE. In addition to itself, VDR has been shown to partner with and transactivate gene expression through members of the RXR family (35,36) and with other as of yet unknown factors (37-39).

VDREs have generally been characterized as a direct repeat of the hexameric sequence PuG(G/T)TCA separated by three nucleotides (40). Nevertheless, there are noted nucleotide binding and transactivating preferences for homo (41) versus heterodimeric (42) VDR complexes within this hexameric motif.

Although there have been many examples of vitamin D responsive sequences that transcriptionally activate genes in recent years (43-45), there have been relatively few VDREs characterized that mediate transcriptional repression through vitamin D (46). The human parathyroid hormone (46) and rat bone sialoprotein (47) genes are perhaps the most frequently cited examples of negative transcriptional regulation through vitamin D. In both cases investigators localized the region of vitamin D responsiveness to promoter DNA sequences of high similarity to the classical VDRE PuG(G/T)TCA half site (40). In the present study, we have characterized the factors and sequences that are important for EGFR downregulation in MCF7, T47D, and BT549 breast cancer cells in response to vitamin D treatment. Starting with the previously identified region of the EGFR promoter which localized the vitamin D responsive region to within 840 nucleotides, we subsequently mapped the primary vitamin D response to a region of the promoter between nucleotide positions -536 and -478 that contains a putative VDRE and transcription factor sp1 binding site. Gel mobility shift studies with nuclear extracts demonstrate the exclusive binding of sp1, or VDR and a heterodimeric partner, to this region of the EGFR promoter. Evidence is presented that suggests that VDR's partner is an as of yet unidentified transcriptional repressor, rather than one of VDR's currently known partners (35,36). Functional reporter assays with a minimal heterologous promoter and wild type -536 to -478 sequence demonstrate that a repressive vitamin D response is in fact mediated through this stretch of the EGFR gene. Similar experiments done with mutations that abolish VDR and/or sp1 binding suggest that the VDR and its partner mediates a repressive effect

on EGFR expression through displacement of sp1. Transfection experiments in BT549 cells suggest that there are additional cell specific factors that mediate the vitamin D repression of EGFR at higher vitamin D concentrations. A model is presented for a mechanism by which one or more cell specific factors interact with the VDR to mediate EGFR repression in response to vitamin D treatment in MCF7, T47D, and BT549 breast cancer cells.

RESULTS

Mapping of the 1,25-dihydroxyvitamin D_3 response to a SacII-Bsu36I fragment of the EGFR promoter.

Our previous transient transfection studies in MCF7, T47D, and BT549 breast cancer cells suggested that vitamin D repression of EGFR expression has a transcriptional mechanism that is mediated through promoter sequences located between nucleotide positions -860 and -20 (31). Examination of the sequence of this region of the EGFR promoter led to the identification of a putative VDRE between nucleotides -531 and -516 with the sequence GGGTCCACAGGGGCA (half sites underlined). So as to assess the possibility that vitamin D repression of EGFR gene expression is mediated through this putative VDRE, serial deletion promoter CAT constructs were generated based on naturally occurring SacII and Bsu36I restriction sites flanking the -531 to -516 region of the EGFR promoter. The constructs pJFCAT 536 and pJFCAT 478 contain EGFR promoter sequence whose 5' ends are -536 and -478, respectively. MCF7 and T47D cells transfected with pJFCAT 536 demonstrated a significant (25-50%) decrease in CAT activity upon 1μM 1,25-dihydroxyvitamin D_3 treatment as compared to CAT activity in untreated transfected controls (figure 1A). In contrast, these same cells transfected with pJFCAT 478 demonstrated no significant change in CAT activity upon 1μM 1,25-dihydroxyvitamin D_3 treatment. Similar results were obtained with 1μM analog C (1α,25-$(OH)_2$-16-en-23-yn-26,27-F_6-vitamin D_3), a vitamin D analog with greater potency (as measured by the ability to induce differentiation and inhibit cellular proliferation), less toxicity (as measured by the ability to stimulate intestinal calcium adsorption and bone calcium mobilization), and greater affinity binding to the vitamin D receptor than 1,25-dihydroxyvitamin D_3 (20,48)(data not shown). These results suggested to us that the region of the EGFR promoter between the SacII and Bsu36I sites (i.e., nucleotide positions -536 and -478), containing a putative

VDRE, were important for mediating the 1,25-dihydroxyvitamin D_3 repressive effect on EGFR expression in MCF7 and T47D cells.

Figure 1B shows the results of transfection of the pJFCAT 536 and pJFCAT 478 constructs in BT549 cells followed by 1,25-dihydroxyvitamin D_3 treatment. Unlike the MCF7 and T47D cells, complete loss of a 1μM 1,25-dihydroxyvitamin D_3 response was not observed with the pJFCAT 478 construct in BT549 cells. Instead, the 40% repression observed with the pJFCAT 840 and pJFCAT 536 constructs was lessened by a factor of 2 with elimination of the sequence between nucleotides -536 and -478. The resulting 20-25% repression seen with the pJFCAT 478 construct at 1μM 1,25-dihydroxyvitamin D_3 treatment was only lost when an additional 333 nucleotides were eliminated as evidenced by results of transfections with the pJFCAT 145 construct. Transfections of the pJFCAT 840, pJFCAT 536, and pJFCAT 478 constructs in BT549 cells followed by treatment with 1,25-dihydroxyvitamin D_3 at a concentration which was 10 fold lower, however, resulted in an effect similar to that observed in MCF7 and T47D cells at the 1μM concentration. Specifically, BT549 cells transfected with pJFCAT 840 or pJFCAT 536 demonstrated a significant (20-30%) decrease in CAT activity upon 0.1μM 1,25-dihydroxyvitamin D_3 treatment, while transfection of the pJFCAT 478 construct demonstrated no significant change in CAT activity with this treatment. Similar results were obtained with 1μM and 0.1μM analog C treatment, respectively (data not shown). The dose-dependent results of these transfection experiments in the BT549 cell line, along with the data obtained in the MCF7 and T47D cell lines, suggested to us that the primary 1,25-dihydroxyvitamin D_3 effect on EGFR expression in breast cancer cells was mediated through sequences located between nucleotides positions -536 and -478 of the EGFR promoter. The identification of a second 1,25-dihydroxyvitamin D_3 responsive region of the EGFR promoter between nucleotides -478 and -145 exclusively in the BT549 cell line, which only repressed at a 10 fold higher concentration of

ligand, suggested to us that it was most likely not essential in mediating EGFR repression by 1,25-dihydroxyvitamin D_3 in these breast cancer cells.

***In vitro* footprinting of factors binding to the -536 to -478 1,25-dihydroxyvitamin D_3 responsive region of the EGFR promoter.**

The results of the transfection experiments with progressive deletions of the EGFR promoter in MCF7, T47D, and BT549 cell lines suggested to us that the region between nucleotides -536 and -478 mediates the primary 1,25-dihydroxyvitamin D_3 repressive effect on EGFR gene expression. To ascertain whether or not the putative VDRE sequence identified between nucleotides -531 to -516 binds protein factor(s) present in the nucleus, *in vitro* DNAse I footprinting was performed using crude nuclear extract and a polymerase chain reaction (PCR) generated fragment spanning the SacII to Bsu36I region of the promoter. Figure 2 shows the results of a footprint done using BT549 nuclear extract and this restriction fragment labeled at the SacII end. Sanger dideoxy sequencing reactions were run in parallel and delinate the areas of protein binding at the nucleotide level. Identical results were obtained with extracts from MCF7 and T47D cell lines (data not shown). The regions of protection evident from this assay are labeled I, II, and IIIa and IIIb. Region I covers the sequence AACTCCTCA, region II the sequence GAACGCCCCT, region IIIa the sequence GGGGCA , and region IIIb GGGTCC. Based on their similarity to known consensus factor binding sites, region I was postulated to be a nuclear receptor half site, region II postulated to be a general transcription factor sp1 binding site, and regions IIIa and IIIb a putative VDRE.

Identification of factors binding to the vitamin D responsive region.

Characterization of factors binding to the EGFR vitamin D responsive region was

accomplished by electrophoretic mobility shift assays (ESMAs). Figure 3 is a representative autoradiogram that demonstrates the results of using crude MCF7 nuclear extract and a radiolabeled SacII to Bsu36I restriction fragment (i.e., nucleotide positions -536 to -478) as a probe in EMSA. Identical results were obtained with MCF7, T47D, and BT549 extracts (data not shown). Upon addition of nuclear extract to labeled probe, an upper and lower complex were formed (lane 2). The location and intensity of the upper and lower complexes were not altered by pretreating the cells with 1,25-dihydroxyvitamin D_3 prior to isolation of nuclear protein, nor by the addition of 1,25-dihydroxyvitamin D_3 at a final concentration of 1μM to the binding reactions (data not shown). The upper complex was specifically competed with 50 fold molar excess of a commercially available sp1 consensus oligonucleotide (lane 3), while the lower complex was specifically competed with a 50 fold molar excess of a consensus VDRE oligonucleotide (lane 4). Neither complex was competed by a 50 fold molar excess of consensus ERE or AP2 oligonucleotides (lanes 5 and 6). Addition of 1μg of a polyclonal antibody directed against transcription factor sp1 resulted in a supershift of the upper complex, but not the lower (lane 7), while addition of 1μg of a polyclonal antibody directed against the VDR resulted in a stabilization and supershift of the lower complex (more apparent upon lighter exposure or longer gel run), but not the upper (lane 8). As controls, incubation of 1μg of the sp1 (lane 12) or VDR (lane 13) antibodies with probe alone resulted in no complex formation. Further, to rule out the possibility that transcription factor sp3 might also bind to this region of the EGFR promoter, since it recognizes the same DNA sequence as sp1 but mediates a repressive rather than an inductive transcriptional response (49), a sp3 antibody was used but failed to recognize the upper (and lower) complex (data not shown). Altogether, these results suggested to us that the upper complex represents transcription factor sp1 binding, while the lower represents binding of the VDR.

Further confirmation of the identity of the binding factors to this region of the EGFR

promoter was accomplished through the use of purified factors sp1, hRXRß, and hVDR in the same EMSA. Incubation of 10ng of purified sp1 with the SacII-Bsu36I probe resulted in the formation of a complex that migrated to a position in the gel equal to that of the upper complex formed by incubation of the probe with crude nuclear extract (lane 9). Incubation of up to 100ng purified hVDR with the SacII-Bsu36I probe resulted in no complex formation unless 1,25-dihydroxyvitamin D_3 was added to the binding mixture (data not shown), at which point the VDR formed a complex with the DNA (presumably a VDR monomer) that migrated to a point below that of the lower complex formed with nuclear extract (see figure 4, lane 2). In contrast, incubation of the probe with 10ng of purified VDR along with 10ng of purified RXRß, which has been shown to be a coregulator of VDR binding and transactivation (35,36), in the presence or absence of 1,25-dihydroxyvitamin D_3, resulted in the formation of a complex that migrated slightly above that observed for the lower complex formed with nuclear extract (figure 3, lane 10). Incubation of 10ng each purified sp1, VDR, and RXRß with probe, in the presence or absence of 1,25-dihydroxyvitamin D_3, resulted in the formation of a complex that migrated above that observed for either of the lower or upper complexes (lane 11).

Since purified VDR alone formed a weakly detectable complex with the probe even when in excess, and only when 1,25-dihydroxyvitamin D_3 was present, it suggested to us that VDR must partner with a factor other than itself to effectively bind to this region of the EGFR promoter. Indeed, when partnered with RXRß the VDR formed a complex with the probe which ran slightly above the lower complex formed with nuclear extract (figure 3, lane 10). However, while specific competition and supershift results indicated that the lower nuclear complex contained the VDR, differences in mobility between the purified VDR/RXRß complex and the lower nuclear complex suggested that it was not composed of a VDR/RXRß dimer. In an effort to identify the factor present

in the nuclear extract that dimerized with the VDR and formed the lower complex, additional experiments were performed with polyclonal antibodies directed against known potential partners of VDR, including the RXRα, RXRß, RXRγ, RARα, RARß, and RARγ. Despite their ability to supershift their cognizant purified receptors when bound to DNA, none of these antibodies recognized the lower complex, suggesting that it was composed of the VDR and an as of yet unidentified partner (data not shown).

Purified VDR and sp1 are capable of binding simultaneously to the SacII-Bsu36I region in the absence of nuclear extract.

Our EMSA results provide evidence that transcription factor sp1 and the VDR with some unknown partner bind to the nucleotide sequences between the SacII and Bsu36I restriction sites in the EGFR promoter. Given that a tertiary complex representing sp1, VDR, and its unknown partner was never seen by EMSA done with nuclear extract as compared to the complex observed with purified sp1, VDR, and RXRß (figure 3, lane 2 versus lane 11), it also suggested that the binding of sp1 was mutually exclusive of the binding of VDR and its unknown partner. Figure 4 is an autoradiogram of an EMSA demonstrating the specificity of VDR and sp1 binding to the EGFR SacII-Bsu36I restriction fragment, as well as their ability to bind to this region simultaneously in the absence of nuclear extract. All binding reactions here with purified VDR were carried out in the presence of 1μM 1,25-dihydroxyvitamin D_3. The incubation of either 10ng of purified VDR or 10 ng of purified sp1 with the DNA probe each resulted in a single shifted band (lanes 2 and 3). When purified VDR and sp1 were incubated together with the probe, a tertiary complex was formed, presumably representing a VDR/sp1 complex (lane 4). When 100 fold molar excess of a consensus DR3 VDRE oligonucleotide competitor was used, the tertiary VDR/sp1 complex was competed

down to just a sp1 complex (lane 5), and when 100 fold molar excess of a sp1 oligonucleotide competitor was used, the tertiary VDR/sp1 complex was competed down to just VDR monomer binding (lane 6). These results provide additional evidence that the VDR binds to this vitamin D responsive region of the EGFR promoter, and further suggests that nuclear extract contains an unidentified VDR partner that prevents simultaneous binding of the VDR and sp1 to this region.

Specific mutations introduced into putative VDR and sp1 binding sites abolish VDR and sp1 factor binding.

While figures 3 and 4 indicate that sp1 and the VDR and some unknown partner bind directly to the SacII-Bsu36I restriction fragment, mutational analysis of this region followed by EMSAs confirmed the importance of specific nucleotide sequences in this binding. The results of the footprint presented in figure 2, along with a knowledge of consensus sequences for VDR and sp1 factor binding sites, allowed us to design a series of point mutations in protected regions II and IIIa and IIIb. Figure 5A shows diagrammatically the wild type, VDRE, sp1, and double VDRE/sp1 mutant sequences used as probes in EMSAs. Footprint protected region II was predicted to be the sp1 binding site and was therefore mutated by introducing a NsiI site over the center of the ACGCCC motif. Protected regions IIIa and IIIb were predicted to encompass a putative VDRE. These were mutated by introducing HindIII and EcoRV sites over the two putative half sites as shown. The double VDRE/sp1 mutant was generated by a combinatorial insertion of all three restriction sites over their respective protected regions.

Figure 5B is a representative EMSA using the sp1, VDRE, and VDRE/sp1 double mutant sequences as probes. When incubated with nuclear extract, wild type probe demonstrated the formation of the upper and lower complexes (lane 2). Use of the VDRE mutant as a probe for

nuclear extract resulted in the loss of binding of the lower complex (lane 4), suggesting that the putative VDRE does in fact bind the VDR and its unknown partner. Lane 6, using the sp1 mutant probe and nuclear extract, demonstrates the loss of upper complex formation, suggesting that region II is in fact a binding site for transcription factor sp1. Finally, use of the double VDRE/sp1 mutant as a probe resulted in loss of both lower and upper complex formation.

So as to further confirm the specificity of the mutations introduced into the SacII-Bsu36I restriction fragment before assessing their functionality in reporter assays, a series of EMSA competition assays were performed. Figure 6A shows the results of competition experiments done with nuclear extract, a labeled consensus VDRE probe, and each of the DNA sequences shown in Figure 5A as unlabeled competitors. Lane 2 shows an uncompeted control band representing a VDR-consensus DNA complex. When increasing molar excess of wild type sequence (lanes 3-5) or the sp1 mutant sequence (lanes 9-11) was used, competition was observed, demonstrating the binding of the VDR to the wild type putative VDRE sequence. When increasing molar excess of VDRE mutant sequence (lanes 6-8) or the double VDRE/sp1 mutant sequence (lanes 13-15) was used, competition was not observed, demonstrating the effectiveness of the mutations introduced to cause loss of VDR binding. In a similar manner, figure 6B shows the results of competition experiments done with a labeled consensus sp1 oligonucleotide probe and each of the DNA sequences as unlabeled competitors. Again, lane 2 shows an uncompeted control band representing a sp1-consensus DNA complex. With increasing molar excess of wild type sequence (lanes 3-5) or VDRE mutant sequence (lanes 6-8) was used, competition was observed, demonstrating the binding of sp1 to the putative sp1 site. When increasing molar excess of sp1 mutant sequence (lanes 9-11) or the double VDRE/sp1 mutant (lanes 12-14) was used, competition was not observed, demonstrating the effectiveness of the sp1 mutation introduced to cause loss of sp1 binding. Figure

6C shows the results of competition experiments done with a labeled wild type SacII-Bsu36I probe and each of the DNA sequences as unlabeled competitors. As expected, increasing molar excess of wild type sequence competed for both the upper and lower complex (lanes 3-5). When increasing molar excess of VDRE mutant sequence was used, competition was only observed for the upper complex (lanes 6-8), and when increasing molar excess of sp1 mutant sequence was used, competition was only observed for the lower complex (lanes 9-11). The double VDRE/sp1 mutant sequence showed no competition for either upper or lower complexes (lanes 13-15). Together, these experiments demonstrated that the mutations introduced specifically caused loss of sp1 and VDR binding, as well as prevented the formation of the upper and lower complexes observed with nuclear extract.

Wild type SacII-Bsu36I sequence confers a vitamin D response upon a heterologous promoter that is lost with mutants, demonstrating the functionality of the putative VDRE and the involvement of sp1.

To determine whether the sequence between the SacII-Bsu36I sites of the EGFR promoter is able to mediate a repressive vitamin D effect on transcription, a CAT construct driven by a minimal heterologous promoter (a TATA box only) was designed for transient transfection in MCF7, T47D, and BT549 cells. The parental construct, named pJFCAT TATA, was generated by subcloning a synthetic TATA box oligonucleotide upstream of the CAT gene in the pJFCAT construct (50) that was the backbone for the reporter constructs used in figure 1. Initial transfection experiments with pJFCAT TATA containing the SacII to Bsu36I region of the EGFR promoter cloned into it resulted in unmeasurable CAT activity in all three cell lines (data not shown). Consequently, to increase basal CAT activity to a level where we could measure any repression

resulting from subcloned wild type and mutant sequences in response to 1,25-dihydroxyvitamin D_3 treatment, we generated a second CAT vector named pJFECAT TATA by subcloning the pJFCAT poly A cassette and the TATA box oligonucleotide into Promega's pCAT enhancer vector which contains the SV40 enhancer downstream of CAT (see materials and methods). Subsequent transfections of this construct containing wild type sequence produced measurable basal activity in T47D and BT549 cells, but not MCF7 cells.

To rule out the possibility that 1,25-dihydroxyvitamin D_3 may mediate a response through sequences in the pJFECAT TATA vector itself, transfections of this construct were performed in BT549 cells followed by 1μM 1,25-dihydroxyvitamin D_3 and analog C treatments. The results of three independent experiments in this cell line demonstrated that vitamin D had no effect on CAT activity when compared to untreated transfected controls (data not shown). We then asked whether the wild type SacII-Bsu36I sequence could mediate a repressive vitamin D response in the context of the pJFECAT TATA vector. Figure 7A shows the average results of transient transfections in T47D cells from three independent experiments using the wild type, mutant VDRE, mutant sp1, and mutant VDRE/sp1 SacII-Bsu36I sequences illustrated in figure 5A subcloned into the pJFECAT TATA vector. Demonstrated is a significant vitamin D repressive response mediated by wild type sequences between the SacII and Bsu36I sites of the EGFR promoter that was abolished upon specific mutation of either the sp1 or the VDR binding sites. The same results were obtained for transfections of the wild type and mutant sequences in BT549 cells followed by 0.1μM 1,25-dihydroxyvitamin D_3 treatment (figure 7B). However, when BT549 transfectants were treated with 1μM 1,25-dihydroxyvitamin D_3, wild type repressive activity was only partially lost upon mutation of sequences shown to bind either the VDR or sp1 (figure 7C). Complete loss of a vitamin D response was only accomplished in BT549 cells at the 1μM treatment level with mutation of both

sp1 and VDR binding sites. Neither mutation of the VDRE nor the sp1 site alone were sufficient to abolish repression as was observed to be the case with T47D cells and treatment of BT549 cells at a vitamin D concentration 10 fold lower. This suggests that a more complex interaction of factors and/or sequences characterizes the mechanism of 1μM vitamin D repression in BT549 cells when compared to the repression observed at the 0.1μM concentration.

Titration studies suggest the presence of a nuclear factor which binds with the VDR and mediates EGFR repression.

Our results suggest the existence of a nuclear factor which partners with the VDR, binds to the region of the EGFR promoter containing the putative VDRE, and is involved in mediating the 1,25-dihydroxyvitamin D_3 repression of EGFR expression at least partially through displacement of sp1. To provide evidence for the existence of this unidentified factor, titration studies were performed using wild type SacII-Bsu36I probe, nuclear extract, and purified sp1 and VDR proteins. Figure 8A shows the results of adding an increasing amount of purified VDR and sp1 proteins to a binding reaction containing a constant amount of nuclear extract and labeled wild type probe. Lane 2 shows the locations of the upper and lower complexes formed by incubation of nuclear extract alone with probe. Lanes 3-9 show the results of adding purified sp1 and purified VDR (at a constant 1:1 ratio) in 10 ng increments, to binding reactions otherwise identical to lane 2. The gradual disappearance of both the lower and upper nuclear complexes in these lanes, and the gradual appearance of an even slower migrating complex, presumably representing the simultaneous binding of sp1 and VDR to wild type sequence, suggests that one or more additional factor(s) must be present in nuclear extract that prevent the simultaneous binding of the VDR and sp1. In figure 8B the purified sp1 and VDR levels were kept at a constant 10ng while the amount of nuclear extract

was increased in 25ng increments. Lanes 5-14 demonstrate the gradual disappearance of the VDR/sp1 complex (seen with the purified protein in lane 4) and the gradual appearance of the upper and lower complexes on the wild type sequence with increasing amount of nuclear extract. As with the inverse experiment, this suggests the existence of one or more nuclear factors that prevent the simultaneous binding of the VDR and sp1.

DISCUSSION

We have previously shown that downregulation of EGFR expression in MCF7, T47D, and BT549 breast cancer cells in response to 1,25-dihydroxyvitamin D_3 treatment occurs at the mRNA and protein levels (31). In the present report we further characterize the repressive response that 1,25-dihydroxyvitamin D_3 treatment has on EGFR gene expression in these cell lines at the molecular level, demonstrating that the primary vitamin D repressive response is transcriptionally mediated through the VDR and a promoter sequence that resembles a VDRE. Using functional reporter assays and DNA-protein binding studies, we have determined that the EGFR promoter does in fact contain a functional VDRE that spans nucleotides 531 to 516 upstream of the translation start site. This VDRE, with a sequence of GGGTCCACAGGGGCA (half sites underlined), demonstrates remarkable similarity to the classical PuG(G/T)TCA DR3 described as the consensus VDRE by Umesono *et.al.* (40).

Initial identification of this VDRE was facilitated by transient transfection of a CAT reporter construct containing 840 nucleotides of the EGFR promoter into MCF7, T47D, and BT549 cell lines followed by 1μM vitamin D treatment. All three cell lines demonstrated vitamin D repression of promoter activity, and progressive deletions of the EGFR promoter allowed for localization of the response to a region between the SacII and Bsu36I restriction sites at nucleotide positions -536 and -478, respectively. In the BT549 cell line, elimination of this 58 nucleotide region of the EGFR promoter did not completely negate the 1μM vitamin D response as it did in MCF7 and T47D cells, but rather only reduced the magnitude of the repression by a factor of 2. This suggested that the SacII to Bsu36I sequence contributes to the overall 1μM vitamin D effect in BT549 cells, but may not exclusively mediate it. Only upon additional deletion of promoter sequence to 145 bases upstream of the translation start site was a complete loss of a 1μM vitamin D effect observed,

suggesting the existence of a second vitamin D responsive region of the EGFR promoter between nucleotide positions -478 and -145 that functions in BT549 cells.

The identification of a common vitamin D responsive region of the EGFR promoter between nucleotides -536 and-478 in MCF7, T47D, and BT549 cell lines lent support to our hypothesis that vitamin D repression of EGFR expression is mediated through a putative VDRE located within this region of the EGFR promoter. In mapping this element, an unexpected result was obtained with the identification of the second 1μM vitamin D responsive region of the EGFR promoter between nucleotides -478 and -145 in BT549 cells only. Consequently, we hypothesized that this second region may mediate a 1μM vitamin D response through cell specific factors that allow utilization of multiple low affinity VDR binding sites located within it. Support for this is suggested by Kato *et.al.* (51) who note vitamin D mediated gene transactivation through widely spaced, directly repeated PuGGTCA elements. Indeed, when the sequence of this second 1μM vitamin D responsive region of the EGFR promoter is examined, many PuGGTCA motifs are apparent (52,53).

Further characterization of the vitamin D responsive region of the EGFR promoter spanning the SacII to Bsu36I sites by *in vitro* footprinting using nuclear extracts from MCF7, T47D, and BT549 cells demonstrated defined regions of protein binding whose sequences were hypothesized to be sp1 and VDR half site binding motifs. While EMSAs with purified sp1 and VDR showed simultaneous binding of theses factors, the use of nuclear extracts demonstrated the specific but mutually exclusive binding of sp1 and the VDR with an unknown partner. Mutations introduced into the putative sp1 and VDR binding sites confirmed the specificity of binding, while transfection of wild type and mutant sequences in the context of a heterologous, minimal promoter driven CAT construct demonstrated the functionality of the VDRE. In T47D cells at 1μM vitamin D, and in BT549 cells at 0.1μM vitamin D, mutation of either the VDRE or the sp1 binding site completely

abolished the vitamin D effect. However, at 1 μM vitamin D, mutation of the VDRE and sp1 binding site were both required in order to completely abolish the vitamin D response in BT549 cells, suggesting once again the involvement of BT549 cell specific factors and possibly other lower affinity VDR binding sites within the SacII to Bsu36I sequence that gain functionality upon a higher concentration of vitamin D. Such a site might actually correspond to footprint protected region I whose sequence resembles a VDRE half site. Binding of proteins to this region was only seen on footprinting, not EMSA. While this could suggest the existence of a lower affinity, unstable DNA-protein interaction characteristic of just such a VDRE half site, it should also be noted that the 3' end of region I was absent in those probes used for EMSAs (due to use of the 3' Bsu36I restriction site), which could potentially eliminate binding at this site.

1,25-dihydroxyvitamin D_3 mediated repression of gene expression is thought to be controlled through several possible mechanisms. Some cases require binding of vitamin D receptor to DNA (44,46,54-56), while other instances suggest that protein-protein interactions are sufficient to mediate a repressive response (57). In both examples there appears to be an interference with or disruption of the transcriptional machinery that results in repression (58) that is thought to occur on three possible levels (59). The first level involves the basal transcription initiation complex. In this case, the VDR has been shown to interact directly with general transcription factor TFIIB both *in vitro* and *in vivo* through a domain separate from its dimerization domain (60,61). Such an interaction could theoretically result in disruption or enhancement of the transcriptional machinery depending upon what other VDR partners, specific cell factors, and/or binding sequences are present. One way of interpreting our finding of a second vitamin D responsive region of the EGFR promoter between nucleotides -478 and -145, which only represses in BT549 cells, and only at a vitamin D concentration of 1μM, might involve binding of the VDR to multiple, low affinity VDRE half-sites

within this region that interferes with the binding of TFIIB to other members of the transcription initiation complex. This is especially probable in light of the fact that the EGFR promoter has numerous transcriptional start sites and VDRE half sites within this region (52,53), the latter of which by themselves have been shown bind the VDR and mediate gene transactivation in other systems (51). The fact that this region of the EGFR promoter only responds at a high vitamin D concentration supports this mechanism of vitamin D gene repression through low affinity VDREs, while its observation only in BT549 cells, and not MCF7 and T47D cells, suggests the involvement of one or more factors specific to BT549 cells. A similar mechanism has been postulated to explain the vitamin D mediated suppression of the rat bone sialoprotein gene through a region of DNA that contains only a series of irregularly spaced VDRE half sites (47). Additionally, it should be noted that EMSAs were performed using BT549 nuclear extract and restriction fragments spanning the region of the EGFR promoter between positions -478 to -145. In each case, extract failed to show binding of a complex having immunoreactivity to the VDR (data not shown). However, when incubated with excess purified VDR and RXRß proteins (i.e., >50 fold excess when compared to the amount needed to see binding to the SacII to Bsu36I restriction fragment), complexes were noted with several of the DNA probes containing VDRE-like half-sites (data not shown). These results further support our speculation that BT549 cell specific factors coordinate the binding of the VDR and other necessary factors to lower affinity VDRE half sites within the -478 to -145 region of the EGFR promoter, and that such binding contributes to the vitamin D repressive effect on EGFR expression at a concentration of 1μM.

A second level of interference may involve VDR interaction with bridging molecules or coactivators that connect upstream elements with the basal transcription complex (62,63). However, in terms of EGFR such a model lacks support as transfection results using the VDRE in

the context of the minimal heterologous promoter demonstrate that additional upstream elements and/or coactivating factors are not required for vitamin D mediated repression in MCF7, T47D, and BT549 cells. Further, experiments performed with the homologous promoter have shown that EGFR promoter sequence upstream of the VDRE can be removed without affecting the level of vitamin D repression seen in reporter assays. A third level of disruption is suggested to involve direct VDR interaction with other nuclear factors that bind upstream elements and mediate transcriptional activation. In terms of the present study, we hypothesize that the identified EGFR promoter VDRE gains functionality through VDR binding and disruption of the transcription process at this third level, which is above that of the basal transcription initiation complex and any intervening coactivator molecules.

The binding of the VDR to vitamin D responsive DNA sequences has mostly been associated with a corresponding activation of gene expression. These "positive" VDREs are comprised of direct repeats of the sequence PuGGTCA or GGTTCA separated by three nucleotides (40,43-45). Recently, it has been suggested that vitamin D is able to mediate repressive responses by binding to "negative" VDREs which differ from the traditional DR3 by a few nucleotides (44). Such differences in the base composition of the VDRE is believed to cause distinctive conformational changes in the VDR and its transactivation domain that result in transcriptional repression rather than activation (44,46). This type of negative hormone response element has been described for the glucocorticoid receptor mediated downregulation of the preopiomelanocortin gene (64). The functional VDRE identified by us in the present study differs from classical upregulatory VDREs by one nucleotide in its 5' half site (underlined): GGGTCC. It has been demonstrated in other reports (41,65) that the last nucleotide in the 5' VDRE half site tends to be an adenine and is found in up to 93% of VDREs that bind the VDR and activate transcription. This suggests that such a

difference is not of minimal importance. The 3' portion of our VDRE half site, with sequence GGGGCA, is identical to the 3' half site of the upregulatory human osteocalcin gene VDRE (43), but once again differs from the canonical PuGGTCA by one nucleotide. Therefore, it is possible that these two nucleotide differences observed between our VDRE and identified upregulatory VDREs accounts in part for the negative regulation.

Additionally, transcriptional repression mediated through the VDRE identified in this report appears to involve functional interference by the VDR of the activity of positive transcription factors that bind to the EGFR promoter, and in particular, positive transcription factor sp1. Transcription factor sp1 has been shown to bind to GC rich regions of the EGFR promoter in this and other studies (52,53), and is known to mediate activation of genes through its glutamine rich domains (66). Herein we have demonstrated through EMSA the mutually exclusive binding of either sp1 or VDR and an unknown partner to the vitamin D responsive region of the EGFR promoter, suggesting that VDR competes with sp1 for binding and therein exerts transcriptional repression. This is supported by our functional data in T47D cells treated with 1μM vitamin D and in BT549 cells treated with 0.1μM vitamin D, showing that mutation of either the VDRE or sp1 sites, which results in loss of corresponding nuclear factor binding as assessed by EMSA, causes a complete loss of a vitamin D response. This strongly suggests that to exert its repressive effect in T47D cells and in BT549 cells at 0.1μM vitamin D, the VDR must first displace bound sp1 and that mere binding of VDR and its heterodimeric partner to the EGFR VDRE is not sufficient. Other demonstrated examples of this type of transcriptional repression mediated through positive transcription factor displacement by the nuclear receptors and their binding partners include thyroid hormone receptor displacement of sp1 in the EGFR promoter (67), VDR disruption of NFATp/AP-1 binding in the interleukin-2 gene (54), and VDR displacement of AP-1 in the osteocalcin gene (56). An alternative hypothesis to the

displacement of sp1 by the VDR is that sp3, a factor related to sp1 but shown to inhibit its function by competitive binding (49), binds to this region of the EGFR promoter and in some fashion acts with the VDR to downregulate transcription. This idea lacks support, however, as an antibody directed against sp3 failed to recognize it as one of the nuclear binding factors in complex with the SacII-Bsu36I restriction fragment in EMSAs even though it was found to be present in nuclear extract by western analysis (data not shown).

Our functional data also supports the assertion that the identified VDRE, by nature of its nucleotide sequence, mediates negative rather than positive transcriptional responses by vitamin D. That is, mutation of the sp1 site, which abolishes sp1 but not VDR binding, does not subsequently allow the VDR to mediate a positive (rather than negative) transcriptional response as it would be predicted to do if the sequence were a classical "positive" VDRE. Instead, it simply negates or reduces the vitamin D effect. So as to rule out the unlikely possibility that vitamin D simply cannot mediate positive transcriptional responses in MCF7, T47D, and BT549 cells for some unknown reason, we transfected a SV40 promoter driven CAT construct containing an upstream osteocalcin VDRE into each of these cell lines and have observed an induction of CAT in response to vitamin D treatment in each case (data not shown). Since the nature of the base substitutions introduced by us to mutate the VDRE in this report were drastic and abolished VDR binding, it may be interesting to assess whether or not more conservative changes in the VDRE nucleotide sequence that maintain VDR binding would result in a vitamin D mediated up- instead of downregulation of EGFR, thus confirming or refuting the functional significance of the identified half-site mismatches in our VDRE.

While our results clearly indicate that transcriptional repression mediated through our identified EGFR VDRE involves the disruption of sp1 from its proximal binding site, additional

functional data in BT549 cells indicates that the VDR is also able to mediate a degree of transcriptional repression in addition to that which is caused by sp1 displacement, at least in this cell line. At a vitamin D concentration of 1μM in BT549 cells, loss of sp1 binding still allows for a repressive, but blunted, vitamin D response in the context of an intact VDRE and vice versa. We speculate that this level of repression seen in the face of lost sp1 binding might involve one or more additional BT549 cell specific factors that operate through protein-protein contacts to disrupt the activation properties of the initiation complex. An explanation for the maintenance of a repressive, but blunted, vitamin D response with mutation of the VDRE might involve the binding of the VDR and/or its partner to other lower affinity VDRE half sites located within the SacII to Bsu36I restriction fragment, such as footprint protected region I, or alternatively may involve protein-protein interactions mediated by cell specific factors.

In terms of VDR's binding partner, it is known that the RXRs (35,36), and under some circumstances, the RARs (68,69) can partner and bind with the VDR to DNA sequences. The function of such VDR partners can vary, but in most cases they serve to increase affinity of the VDR for VDREs and enhance transactivation potential (35,36). In the present study we have noted that the VDR binds to the identified VDRE with a nuclear protein that shows no immunoreactivity to antibodies raised against RXRα, RXRß, RXRγ, RARα, RARß, and RARγ (data not shown). Based on binding site selection studies, Colnot *et.al.* (65) have determined that sequences which bind the VDR:RXR heterodimer with the highest affinity tend to be positive VDREs (which have an A rather than a C as the last nucleotide in their 5' half site), suggesting that transcriptional repression mediated by vitamin D may not involve heterodimerization of the VDR with RXR. Additionally, in their characterization of the VDR dimer that binds to the VDRE in the human osteocalcin gene (whose 3' half site is identical to the 3' half site of the VDRE we report here for the EGFR gene,)

Jaaskelainen *et.al.* (56) conclude that RXR is not a component. It is interesting to note that while we were able to demonstrate binding of a VDR:RXR heterodimer to our VDRE using purified proteins, a parallel experiment performed with crude nuclear extract demonstrated the formation of a different VDR complex. Therefore we postulate that the VDRE identified in this study gains specific repressive, rather than inductive, functionality through a unique sequence that favors VDR binding as a heterodimer with an as of yet unidentified nuclear factor.

The binding properties of the nuclear factor postulated to dimerize with the VDR and bind to our VDRE were explored in titration experiments. While it is interesting that an excess of purified VDR and sp1 proteins can result in the loss of the nuclear complex containing the VDR and its unknown partner, it is even more intriguing to note the corresponding appearance of a slower migrating complex containing both sp1 and VDR proteins. This sp1/VDR complex was also seen with EMSA using purified factors only, and was found to be disruptable with the addition of increasing amounts of nuclear extract. Its transactivation properties, if any, may be functionally significant with regard to the EGFR upregulation seen in response to vitamin D treatment in the BT-20 breast cancer cell line by Desprez *et.al.* (70) and Falette *et.al.* (71), since it has been demonstrated that sp1 and the VDR can act synergistically in vivo (72). In terms of the BT474 cell line, which in previous studies we have shown upregulates EGFR in response to vitamin D treatment (31), such a complex was never seen by us in EMSAs. Instead, identical complexes were seen with MCF7, T47D, BT549, and BT474 nuclear extracts (data not shown), suggesting that the VDR/sp1 dimer is not functional in these cells through direct binding to the SacII-Bsu36I region of the EGFR promoter. In BT474 cells the observed positive vitamin D mediated regulation of EGFR expression may be due to cell specific factors which coordinate transcriptionally productive protein-protein interactions after VDR DNA binding.

Given this, we have postulated a model in figure 9 which attempts to explain the molecular details surrounding vitamin D mediated repression of EGFR expression in MCF7, T47D, and BT549 breast cancer cells. We propose that the repression pathway is initiated through ligand activation of the VDR, followed by subsequent dimerization with an unknown partner and binding to the negative VDRE spanning nucleotides -531 to -516 of the EGFR promoter. Presumably through a combination of unproductive transcriptional conformational changes in the VDR's transactivation domain brought about by this heterodimerization and/or binding to a "negative" VDRE, displacement of transcription factor sp1 from its proximal binding site then occurs and results in repression by disruption of functional sp1 interactions with the rest of the transcription machinery. Such a disruption may be mediated by the VDR and its partner directly, or through additional factors that coordinate protein-protein contacts. Additionally, in BT549 cells we propose that there is also the direct interaction of the VDR with one or more cell specific factors that allows for recognition and activation of VDRE half sites within a second vitamin D responsive region of the EGFR promoter.

MATERIALS AND METHODS

Plasmid Constructions

All reporter plasmids used were derivatives of pJFCAT which contains a poly A trimer cassette subcloned upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene that effectively blocks any read-through transcription initiated on non-specific plasmid sequences (50). Since the EGFR promoter contains multiple transcription initiation start sites (52,53), all nucleotide positions referred to in this report are relative to the start of translation for the EGFR gene.

We have previously reported on the origin of pJFCAT 840 (31), containing 840 bases of the EGFR promoter between BglII and SacI restriction sites, or between nucleotide positions -860 and -20, respectively. To generate pJFCAT 536, the SacII to SacII fragment of the EGFR promoter spanning positions -536 to -423 was first subcloned into the corresponding site of Bluescript II KS+ in the forward orientation. This clone was then cut at the BamHI polylinker site and the EGFR Bsu36I site (at position -478) to obtain a 83 bp fragment. This 83 bp fragment was then subcloned into the corresponding sites of pJFCAT 840, resulting in the net loss of EGFR sequence between -860 and -536. pJFCAT 478 was obtained by removal of the EGFR promoter sequence between BamHI and Bsu36I sites of pJFCAT 840 and subsequent ligation of klenow blunted ends. pJFCAT 145 was obtained by removal of the EGFR promoter sequence between BamHI and the most 3' promoter NotI site of pJFCAT 840, and subsequent ligation of klenow blunted ends.

For minimal heterologous promoter constructs, Promega's (Madison, WI) pCAT enhancer vector, which contains a SV40 enhancer subcloned downstream of CAT, was used as the backbone plasmid to generate pJFECAT TATA. The HindIII fragment of pJFCAT containing the poly A trimer cassette was inserted into the corresponding polylinker site of the pCAT enhancer vector. In addition to the poly A sequence, this added a BamHI site into the polylinker region of the pCAT

enhancer vector, 5' to the SalI site. A synthetic TATA box sequence flanked by a 5' Bsu36I site and a 3' ClaI site was obtained by annealling sense and antisense oligonuclotides of the sequence GCAAGCCCTCAGGTATAAAACCATCGATGGAAGC. This TATA box sequence was then cut with ClaI and subcloned into the SmaI and ClaI sites of Bluescript II KS+ to allow for confirmation of a single copy insertion and annealing of correct sense and antisense products by sequencing (73). The BamHI to SalI fragment of this subclone containing the TATA box was then ligated into the corresponding sites of the pCAT enhancer vector containing the poly A trimer cassette, keeping it in the forward orientation, to generate pJFECAT TATA.

Wild type EGFR promoter sequence between the SacII and Bsu36I sites (positions -536 to -478) was isolated from the Bluescript II KS+ subclone containing the SacII to SacII sequence (described above) by BamHI and Bsu36I digestion and subcloned into the corresponding sites of pJFECAT TATA to create the "wild type" pJFECAT TATA construct. pJFECAT TATA containing the sp1 mutant sequence was generated by removal of wild type sequence in the latter clone with SacII and Bsu36I restriction enzymes and replacement with sp1 mutant sequence (see below) cut with these same 5' and 3' flanking restriction enzymes. VDRE and double VDRE/sp1 mutant containing pJFECAT TATA constructs were generated from ligation of blunt 5' and Bsu36I cut 3' DNA fragments into 3' Bsu36I and 5' klenow blunted NotI sites found within the pJFECAT TATA construct.

Site Directed Mutagenesis

Mutations were introduced into putative nuclear factor binding sites by PCR amplification (74) of EGFR wild type SacII to klenow blunted Bsu36I sequence cloned into the SacII and SmaI sites of Bluescript II KS+. Blunting of the Bsu36I site and subsequent ligation into the SmaI site

of Bluescript II KS+ regenerated the Bsu36I site. Using this template with a combination of synthetic oligonucleotides incorporating mismatched bases and commercially available universal sequencing primers, the sp1, VDRE, and double VDRE/sp1 mutants were obtained. The identity of all PCR products was confirmed by restriction digestion with unique sites inserted into each mutant, as well by sequencing (73) of fragments after subcloning into Bluescript II KS+. To generate the sp1 mutant, a synthetic oligonucleotide of sequence GCGGTGCCCTGAGGAGTTAATTTCCCGAGAGatGCaTTCCCAGCACTG (mismatched bases in lowercase) was used with the M13 reverse primer. This mutant oligo contains a 3' Bsu36I site to facilitate subcloning and a NsiI site over the sp1 binding region to facilitate identification from wild type DNA. The VDRE mutant was generated through two separate PCR reactions. In the first, a mutant oligo of sequence GCAAGTCCGCGGCGACCGaagCttGACGGGCAGTGCTG (mismatched bases in lowercase) was used with the T7 sequencing primer. This mutant oligo contains a 5' SacII site to facilitate subcloning and a HindIII site over the 5' VDRE half site to facilitate identification from wild type DNA. After PCR, the product was cut with SacII, subcloned into Bluescript II KS+ SacII and SmaI sites in the reverse orientation, and used as a template in a second PCR reaction with a mutant oligo of sequence GACCGAAGCTTGAtatctAGTGCTGGGAAC (mismatched bases in lowercase) and the M13 reverse primer. This second mutant oligo introduced a EcoRV site over the 3' VDRE half site to facilitate the identification of the mutant PCR product. To generate the double VDRE/sp1 mutant, the VDRE mutant sequence was cut with Bsu36I after PCR and subcloned in the forward orientation into the SmaI site of Bluescript II KS+. The mutant sp1 oligo described above was then used with the T7 sequencing primer to amplify the intervening sequence.

Cell Culture and Transient Transfections

Standard culture conditions consisted of phenol red IMEM supplemented with 10% heat-inactivated fetal bovine serum, 37°C humidified atmosphere of 95% air-5% CO_2, and media change every 2-4 days. For each transfection of a 78.5mm^2 dish, cells were grown under standard culture conditions to 70% confluence. 30 minutes prior to transfection, 10μg of CsCl banded DNA was incubated with 20μl of lipofectamine (GIBCO/BRL) in 1 ml serum free IMEM at room temperature. 2 mls IMEM with 10% FBS and 1 ml serum free IMEM was then added per reaction mix, so that the final serum concentration was no more than 5%. Dishes were then incubated under standard conditions for 16-20 hours at which time cells were rinsed 2X with 1X PBS, trypsinzed, pelleted, resuspended, mixed thoroughly, and replated in equal numbers to control for any differences in transfection efficiency from one plate to the next. Upon replating, cell were either given IMEM with 10% FBS and 1,25-dihydroxyvitamin D_3 or analog C, or IMEM with 10% FBS and an equivalent volume of vehicle (100% ethanol). Treatments were carried out for 48-60 hours under standard culture conditions, followed by harvesting. Cell pellets were lysed by freeze/thawing, and the concentration of the protein in the lysate determined in duplicate by the Bradford assay (75). Equal amounts of protein (between 100-500μg depending upon the cell line used) were then incubated at 37°C for 2 hours with 0.125μCi ^{14}C-chloramphenicol and 0.5mM acetyl CoA, and then extracted with ethyl acetate. Samples were spotted onto thin layer chromatography plates, run in a 95:5 chloroform:methanol tank, and quantitated by comparison of phosphorimager determination of percent conversion of chloramphenicol to acetylated forms in treated versus untreated samples.

***In Vitro* DNAse I Footprinting**

Crude nuclear extract was prepared from 1μM 1,25-dihydroxyvitamin D_3 treated and

untreated MCF7, T47D, and BT549 cell lines by the method of Dignam *et.al.* (76). Generation of a singly end labeled DNA probe was accomplished through PCR amplification of wild type EGFR SacII to Bsu36I sequence subcloned in the reverse orientation into SacII and SmaI sites of the Bluecript II KS+ plasmid. γ-^{32}P ATP and T4 polynucleotide kinase was used to label the T7 sequencing primer, followed by inactivation of the kinase by heating to 65°C for 1 hour. Labeled T7 primer was then added to unlabeled M13 reverse primer to PCR amplify the Bluescript II KS+ template containing wild type SacII to Bsu36I sequence. Purification of the PCR product from free nucleotides and unincorporated primers was accomplished by running it through a sepharose column after removing excess Bluescript II KS+ polylinker sequence from the unlabeled end by EcoRI digestion. For each sample, preincubation of nuclear extract (10μg) and nonspecific *E. coli* competitor DNA (0.5μg) was performed at room temperature for 20 minutes in a buffer containing 88mM KCl, 10mM HEPES pH7.9, 12% glycerol, 10mM Tris pH8.0, and 1mM DTT. Following this, radiolabeled probe was added (10,000 dpm/sample or 1ng/sample) to the extract and *E. coli* DNA (30 μl total reaction volume) mix and allowed to incubate at room temperature for an additional 20 minutes. Control reactions were set up in parallel that contained only *E. coli* DNA and BSA.

For DNAse I digestion, the above reactions containing probe, protein, and *E. coli* DNA were incubated with various concentrations of DNAse I (Promega) spanning two orders of magnitude (from 0.1 to 10 units so as to achieve maximum sensitivity) for two minutes at room temperature. The reactions were stopped by adding an excess of SDS and EDTA, phenol:chloroform extracted, and ethanol precipitated. Samples were then denatured in 80% formamide by heating to 90°C and run on a 6% denaturing polyacrylamide gel along with Sanger dideoxy sequencing reactions (73). The sequencing reactions were produced by using the T7 sequencing primer and the same plasmid

DNA that was used as a template in PCR to generate the singly end labeled probe.

Electrophoretic Mobility Shift Assays

Nuclear protein extracts were prepared as described above for use in footprint experiments. For each sample, nonspecific *E. coli* DNA competitor (0.5μg) was preincubated for 20 minutes at room temperature with nuclear extract (10 μg) or purified protein (10ng) and BSA (100-500ng) in the same buffer that is described above for footprinting. Purified VDR was purchased from PanVera Corporation (Madison, WI), purified sp1 from Promega, and purified RXRß from Affinity Bioreagents (Neshanic Station, NJ). Preincubation of either crude nuclear extract or purified protein was followed by an additional 20 minute room temperature incubation with a radiolabeled DNA restriction fragment or commercially available oligonucleotide (10,000 dpm/sample). A consensus double stranded sp1 oligo of sequence ATTCGATCGGGGCGGGGCGAGC was obtained from Promega, a DR3 (VDR) element from the annealling of the sense and antisense strands of a 21 nucleotide sequence GATCGGGTCAGTGAGGTCAGC, an AP-2 oligo of sequence GATCGAACTGACCGCCCGCGGCCCGT from Promega, and an ERE oligo from the annealling of the sense and antisense strands of a 53 nucleotide sequence GGGGGTCAGCTGTGCCCCGGTCGCCGAGTGGCGAGGAGGTGACGGTAGCCGCC. Radiolabeling of probes was accomplished by γ-^{32}P ATP and T4 polynucleotide kinase, followed by sepharose column purification as described for footprinting. In cases of antibody or DNA competitor treatments, antibody (1μg) or DNA competitor (10, 50, or 100 fold molar excess) were added to the reaction mix for 20 additional minutes at room temperature prior to the addition of probe. The polyclonal anti sp1 and sp3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the polyclonal anti-VDR antibody was purchased from Affinity Bioreagents.

Polyclonal antibodies against α, ß, and γ forms of RXR were obtained from Santa Cruz Biotechnology, while polyclonal antibodies against the corresponding forms of RAR were obtained from Dr. Wayne Vedeckis, Louisiana State University Medical Center, New Orleans, Louisiana. After incubation, samples were electrophoresed on a 6% non-denaturing polyacrylamide gel and visualized by autoradiography.

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FIGURE LEGENDS

Figure 1. Mapping of 1,25-dihydroxyvitamin D_3 Responsive Sequences within the EGFR Promoter.

A, Shown schematically are the pJFCAT 840, 536, and 478 constructs encompassing various lengths of the EGFR promoter. Horizontal arrowhead represents the first major *in vivo* start of transcription. Numbering is relative to the start of translation. These constructs were generated based on naturally occurring restriction sites (see text). The results of transient transfection of these constructs in MCF7 and T47D cells followed by 1μM 1,25-dihydroxyvitamin D_3 treatment are shown graphically directly to the right of the plasmid maps. The data presented are the average of at least three independent experiments comparing the percent conversion of chloramphenicol to acetylated forms in treated versus untreated samples as determined by phosphorimaging of TLC plates. Vertical dashed line indicates the 100% (control, untreated) activity level. Error bars represent the standard deviation of the data. B, As in (A) except that BT549 cells were transfected with pJFCAT 840, 536, 478, and 145 constructs followed by treatment with 1 and 0.1μM 1,25-dihydroxyvitamin D_3.

Figure 2. *In vitro* DNAse I Footprinting of the EGFR Promoter SacII-Bsu36I Vitamin D Responsive Sequence.

Representative footprint of the PCR generated SacII-Bsu36I restriction fragment of the EGFR promoter spanning nucleotide positions -536 to -478, singly labeled at the SacII end. Sanger dideoxy sequencing reactions were run in parallel in GATC lanes to delineate protected regions of the DNA probe at the nucleotide level. Samples in lanes 1 and 2 designated "- protein" contain 10μg BSA and no extract, while those labeled "+ protein" contain 10μg crude BT549 nuclear extract. DNAse I was used at a concentration of 0.3 units/reaction in lanes 1 and 3, and at a concentration

of 0.6 units/reaction in lanes 2 and 4. Protected regions are indicated in brackets at the extreme right and are labeled I, II, IIIa and IIIb.

Figure 3. EMSA Demonstrates Binding of VDR and sp1 Nuclear Proteins to the SacII-Bsu36I Restriction Fragment.

Representative EMSA using the radiolabeled wild type SacII-Bsu36I restriction fragment from the EGFR promoter shown to contain a vitamin D responsive sequence and crude BT549 nuclear extract (lanes 2-8), or purified VDR, RXRß, and sp1 proteins (lanes 9-11). Indicated by arrowheads are upper and lower complexes formed upon incubation of nuclear extract with probe. Asterisk (*) marks the locations of supershifted upper complex in lane 7, and lower complex in lane 8, in the presence of anti-sp1 and anti-VDR antibodies, respectively. Free probe alone is shown in lane 1, and antibody incubation with probe alone in lanes 12 and 13. See accompanying grid for specific lane contents.

Figure 4. EMSA Demonstrates Specific Binding of Purified VDR and sp1 Proteins to the SacII-Bsu36I Restriction Fragment.

Representative EMSA using the radiolabeled wild type SacII-Bsu36I restriction fragment from the EGFR promoter shown to contain a vitamin D responsive sequence and purified VDR and sp1 proteins. Indicated by arrowheads are the formation of a VDR/sp1 complex in lane 4, sp1 complex in lane 3 (and in lane 5 in the presence of a specific VDR competitor), and a VDR monomer complex in lanes 2, 4, and 6. Free probe alone is shown in lane 1. See accompanying grid for specific lane contents.

Figure 5. EMSA Shows Loss of VDR and sp1 Binding Upon Mutation of Wild Type SacII-Bsu36I Sequence.

A, Nucleotide sequences of wild type, VDRE mutant, sp1 mutant, and VDRE/sp1 double mutant fragments used in subsequent binding and functional assays. Indicated are VDRE and sp1 footprint protected areas illustrated in figure 2 as regions IIIa & IIIb and II, respectively. The location of flanking 5' SacII and 3' Bsu36I restriction sites are noted in all sequences. The inserted NsiI site is shown for the sp1 and double VDRE/sp1 mutants, while the inserted HindIII and EcoRV sites, over 5' and 3' VDRE half sites, respectively, are also shown for the VDRE and double VDRE/sp1 mutants. B, EMSA using radiolabeled wild type (lanes 1 and 2), VDRE mutant (lanes 3 and 4), sp1 mutant (lanes 5 and 6), and VDRE/sp1 double mutant (lanes 7 and 8) sequences and BT549 nuclear extract. Arrowheads indicate the presence of the upper and lower nuclear complexes binding to wild type probe in lane 2, loss of binding of lower complex to VDRE mutant probe in lane 4, loss of binding of upper complex to sp1 mutant probe in lane 6, and loss of both complex binding to VDRE/sp1 double mutant probe in lane 8. Free probes alone are shown in lanes 1, 3, 5, and 7. See text for details.

Figure 6. Specific Competition Occurs through Wild Type and Mutant VDRE and sp1 Sequences.

A, Representative EMSA using a radiolabeled consensus DR3 (VDRE) probe and BT549 nuclear extract. Free probe (P) and uncompeted control (C) lanes are numbered 1 and 2. Indicated by the arrowhead is the binding of a VDR complex to the probe. Competition at 10, 50, and 100 fold molar excess with wild type (lanes 3-5), VDRE mutant (lanes 6-8), sp1 mutant (lanes 9-11), and double VDRE/sp1 mutant (lanes 12-14) sequences are indicated on the grid at the top. B, As in (A) except that a radiolabeled consensus sp1 probe was used and a sp1 complex was competed. C, As

in (A) except that the radiolabeled SacII-Bsu36I probe was used and upper and lower nuclear complexes were competed.

Figure 7. The Identified VDRE is Functional in the Context of a Minimal Heterologous Promoter and Requires an Intact sp1 Site for Full Activity.

A, The results of transient transfection of the pJFECAT TATA construct containing wild type, VDRE mutant, sp1 mutant, and double VDRE/sp1 mutant sequences in T47D cells are shown graphically. The data presented are the average of three independent experiments comparing the percent conversion of chloramphenicol to acetylated forms in 1μM vitamin D treated versus untreated samples as determined by phosphorimaging of TLC plates. Horizontal dashed line indicates the 100% (control, untreated) activity level. Error bars represent the standard deviation of the data. B-C, As in (A), except that BT549 cells were transfected and treated with 0.1μM (B) and 1μM (C) vitamin D.

Figure 8. Titration of Crude Nuclear Extract and Purified sp1 and VDR Proteins Demonstrate the Presence of an Unknown Nuclear VDR Binding Partner to the VDRE.

A, Representative EMSA done using the radiolabeled SacII-Bsu36I probe, a constant 10ng of purified VDR and 10ng of purified sp1, and increasing amounts of BT549 nuclear extract. Arrowheads indicate the presence of a sp1/VDR complex in lanes 4-9, and the gradual appearance of sp1 (upper) and VDR (lower) nuclear complexes with increasing amounts of BT549 crude extract in lanes 6-14. The binding of purified VDR and sp1 proteins in the absence of nuclear extract is shown in lanes 2-4. Free probe alone is shown in lane 1. See text for details. B, As in (A) except that the amount of nuclear extract was held constant at 250ng while increasing amounts of purified

VDR and sp1 proteins were added. Left arrowheads indicate the presence of sp1 (upper) and VDR (lower) nuclear complexes in control (C) and sample lanes 3-7. Right arrowhead indicates the gradual appearance of a sp1/VDR complex in lanes 5-9 with increasing amounts of purified sp1 and VDR proteins. Free probe (P) alone is shown in lane 1. See text for details.

Figure 9. Schematic Model Illustrating a Possible Mechanism of Vitamin D Mediated Repression of EGFR Expression in MCF7, T47D, and BT549 Cells.

Indicated are conditions of basal expression from the EGFR promoter mediated by transcription factor sp1, possible coactivator molecules, and the components of the RNA polymerase II (RNAP II) initiation complex followed by the events speculated to characterize the vitamin D mediated repressive response in MCF7, T47D, and BT549 cells. At the 1μM concentration, the utilization of multiple lower affinity VDRE half sites presumably through the function of a cell specific factor only active at high concentrations of vitamin D, is also shown. At the bottom is a legend listing the identity of the factors involved. See text for details.

FIGURE 1A

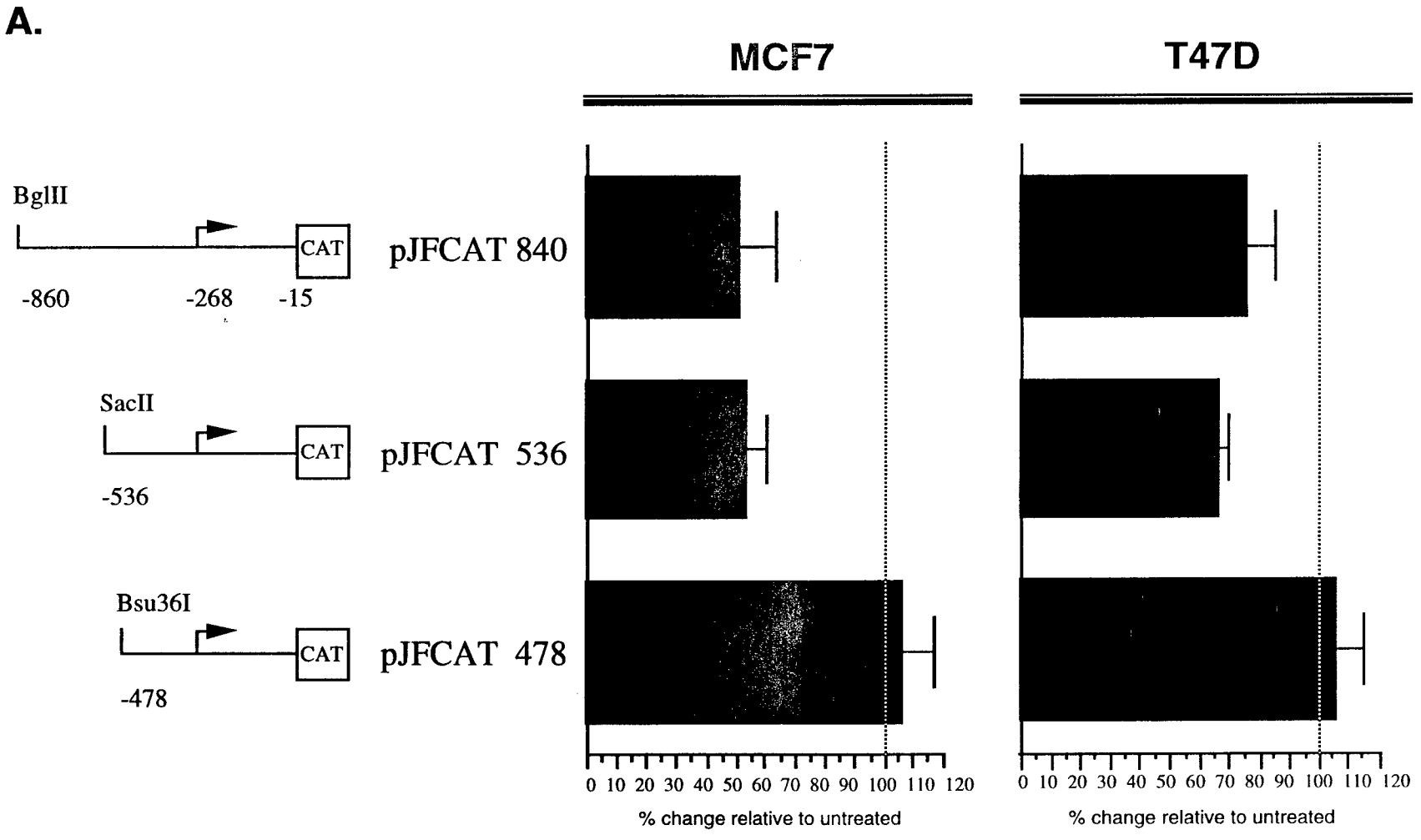
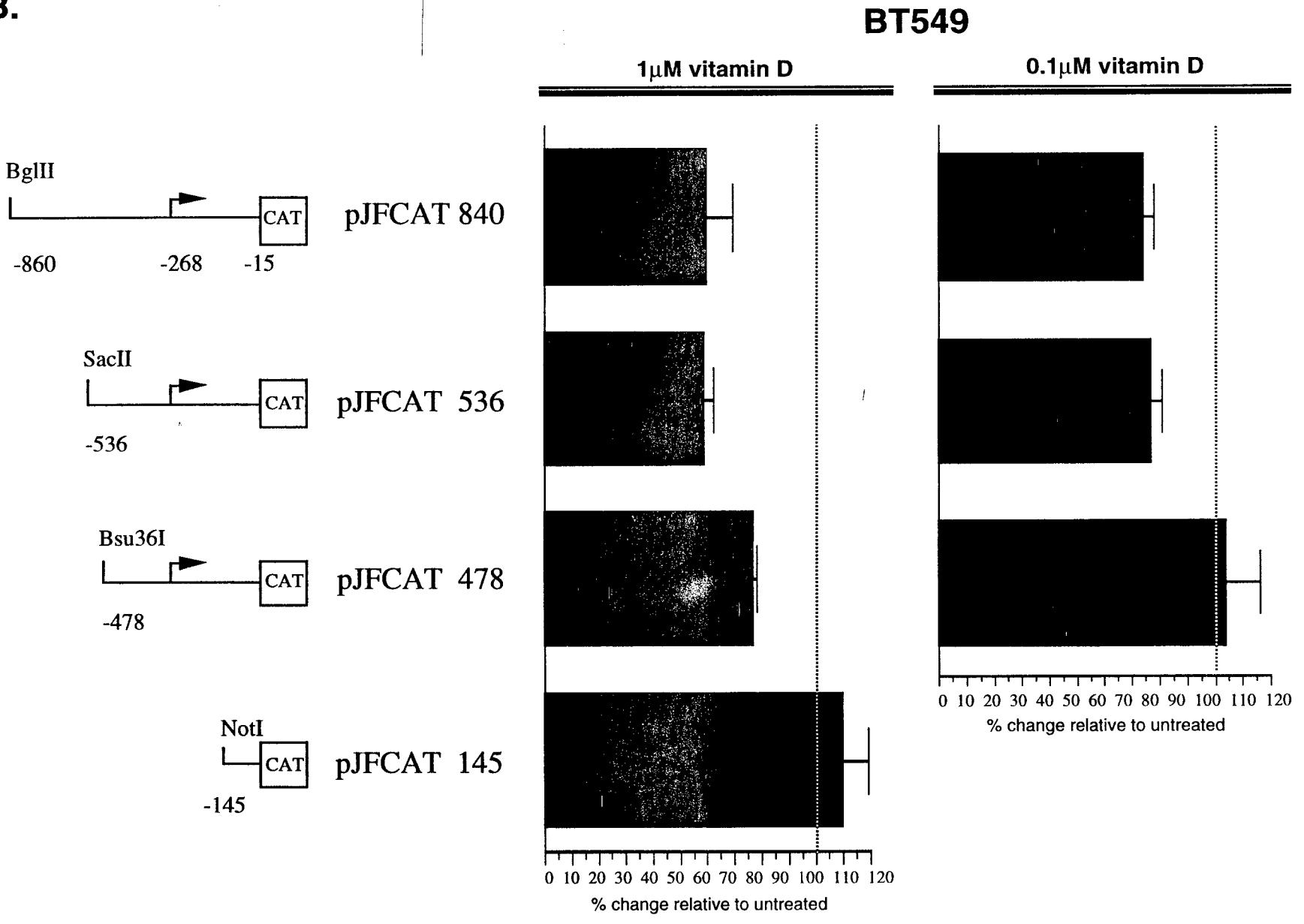

A.
MCF7
T47D
BglII
CAT
-860
-268
-15
pJFCAT 840
SacII
CAT
-536
pJFCAT 536
Bsu36I
CAT
-478
pJFCAT 478
0 10 20 30 40 50 60 70 80 90 100 110 120
% change relative to untreated
0 10 20 30 40 50 60 70 80 90 100 110 120
% change relative to untreated

FIGURE 1B

B.


BglII
CAT
pJFCAT 840
-860
-268
-15
SacII
CAT
pJFCAT 536
-536
Bsu36I
CAT
pJFCAT 478
-478
NotI
CAT
pJFCAT 145
-145
BT549
1μM vitamin D
0.1μM vitamin D
0 10 20 30 40 50 60 70 80 90 100 110 120
% change relative to untreated
0 10 20 30 40 50 60 70 80 90 100 110 120
% change relative to untreated

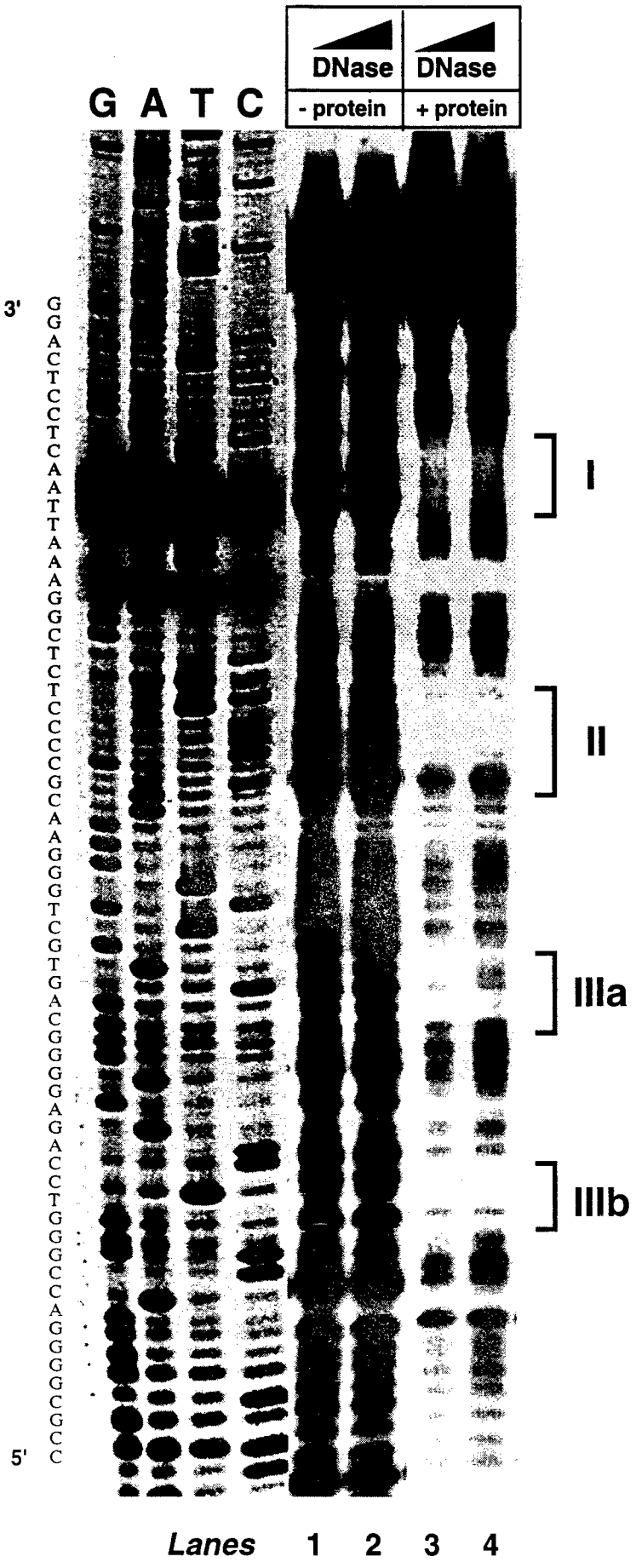

G A T C
DNase
- protein
DNase
+ protein
3'
GGACTCCTCAATTAAAGGCTCTCCCCGCAAGGGTCGTGACGGGGAGACCTGGGCCAGGGGCGCC
5'
I
II
IIIa
IIIb
Lanes 1 2 3 4

FIGURE 2

FIGURE 3

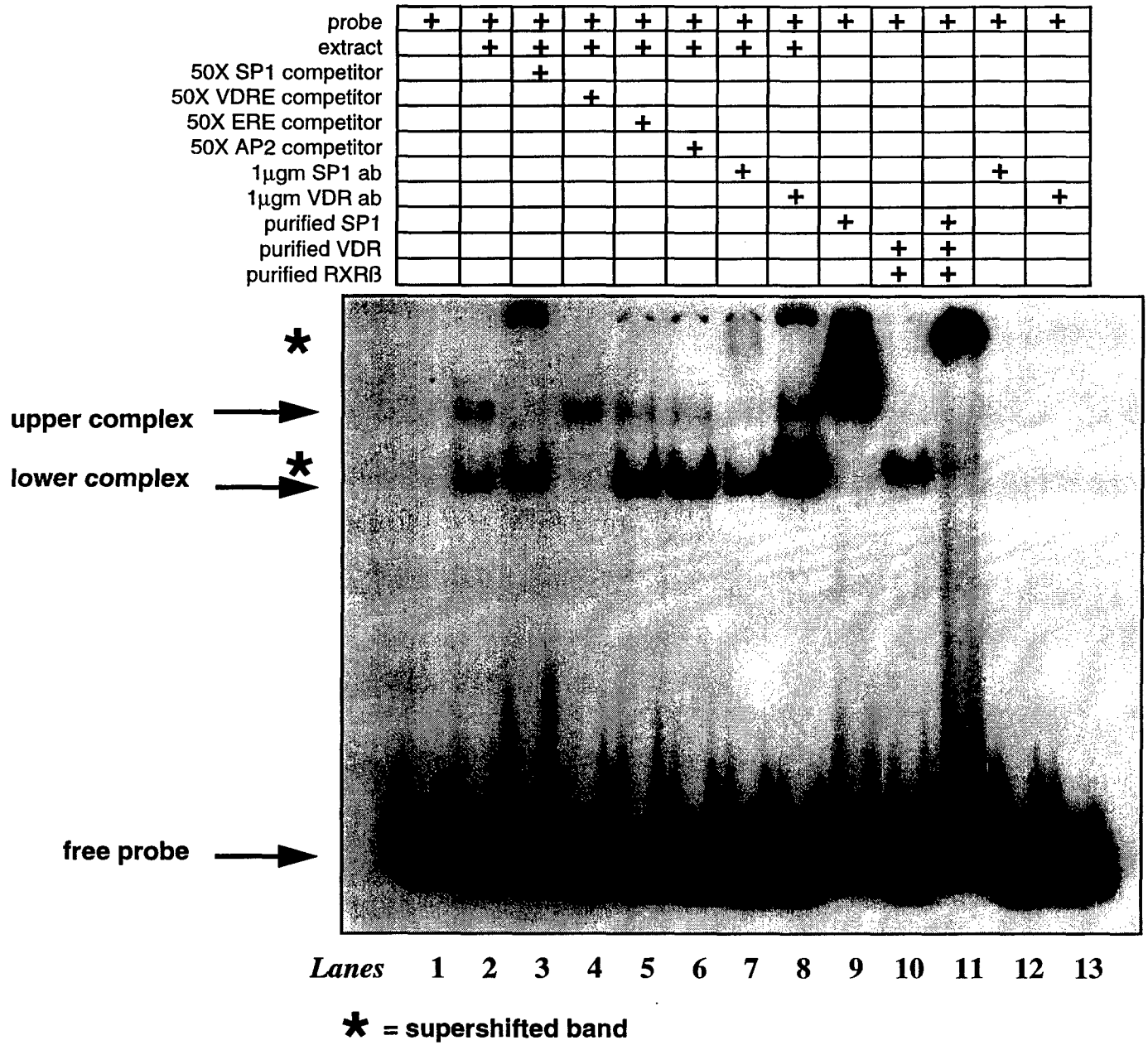
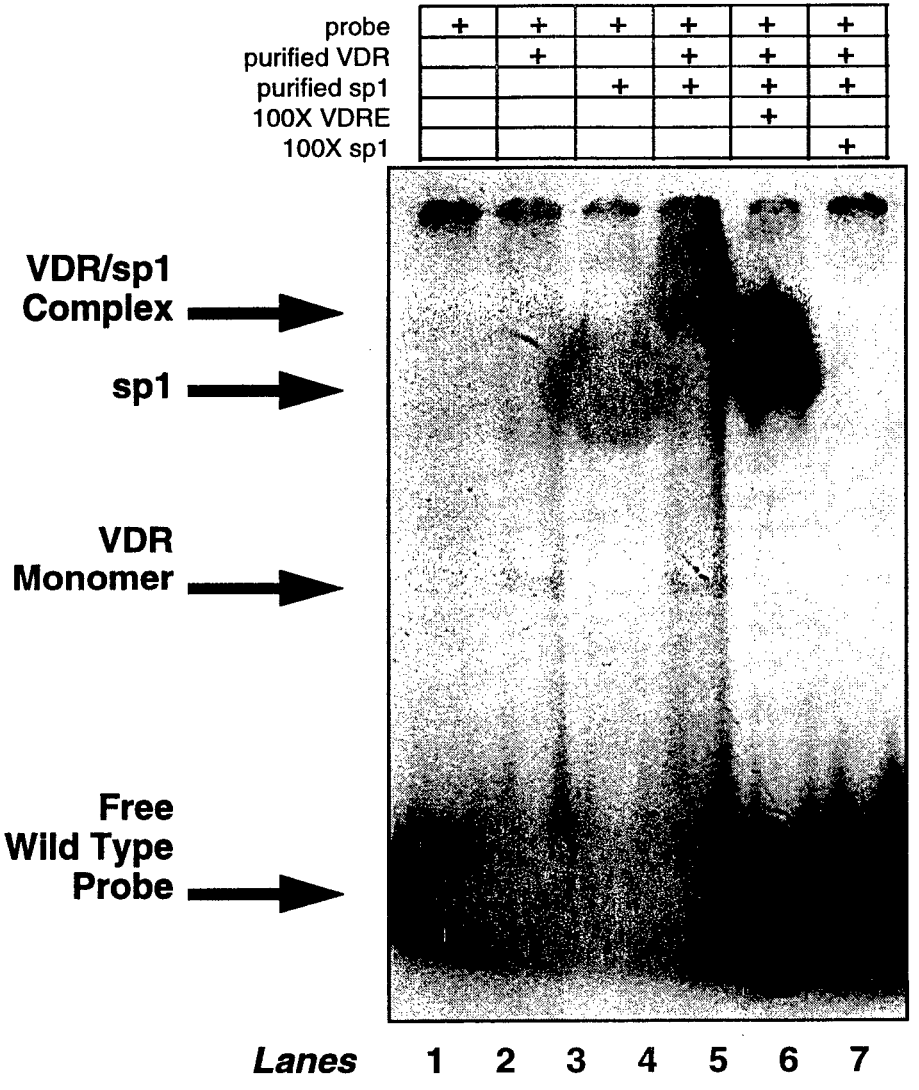

probe
extract
50X SP1 competitor
50X VDRE competitor
50X ERE competitor
50X AP2 competitor
1µgm SP1 ab
1µgm VDR ab
purified SP1
purified VDR
purified RXRß
*
upper complex
lower complex
*
free probe
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13
* = supershifted band

FIGURE 4


probe
purified VDR
purified sp1
100X VDRE
100X sp1
VDR/sp1 Complex
sp1
VDR Monomer
Free Wild Type Probe
Lanes 1 2 3 4 5 6 7

A. **Wild Type**

5' 3'

SacII Bsu36I

GGGGACCGGGTCCAGAGGGGCAGTGCTGGGAACGCCCCTCTCGGGAAATTAACTCC

VDRE SP1 Region I

VDRE Mutant

SacII HindIII EcoRV Bsu36I

GGGGACCGAAGCTTGATATCTAGTGCTGGGAACGCCCCTCTCGGGAAATTAACTCC

SP1 Mutant

SacII NsiI Bsu36I

GGGGACCGGGTCCAGAGGGGCAGTGCTGGGATGCATCCTCTCGGGAAATTAACTCC

VDRE/sp1 Mutant

SacII HindIII EcoRV NsiI Bsu36I

GGGGACCGAAGCTTGATATCTAGTGCTGGGATGCATCCTCTCGGGAAATTAACTCC

B.

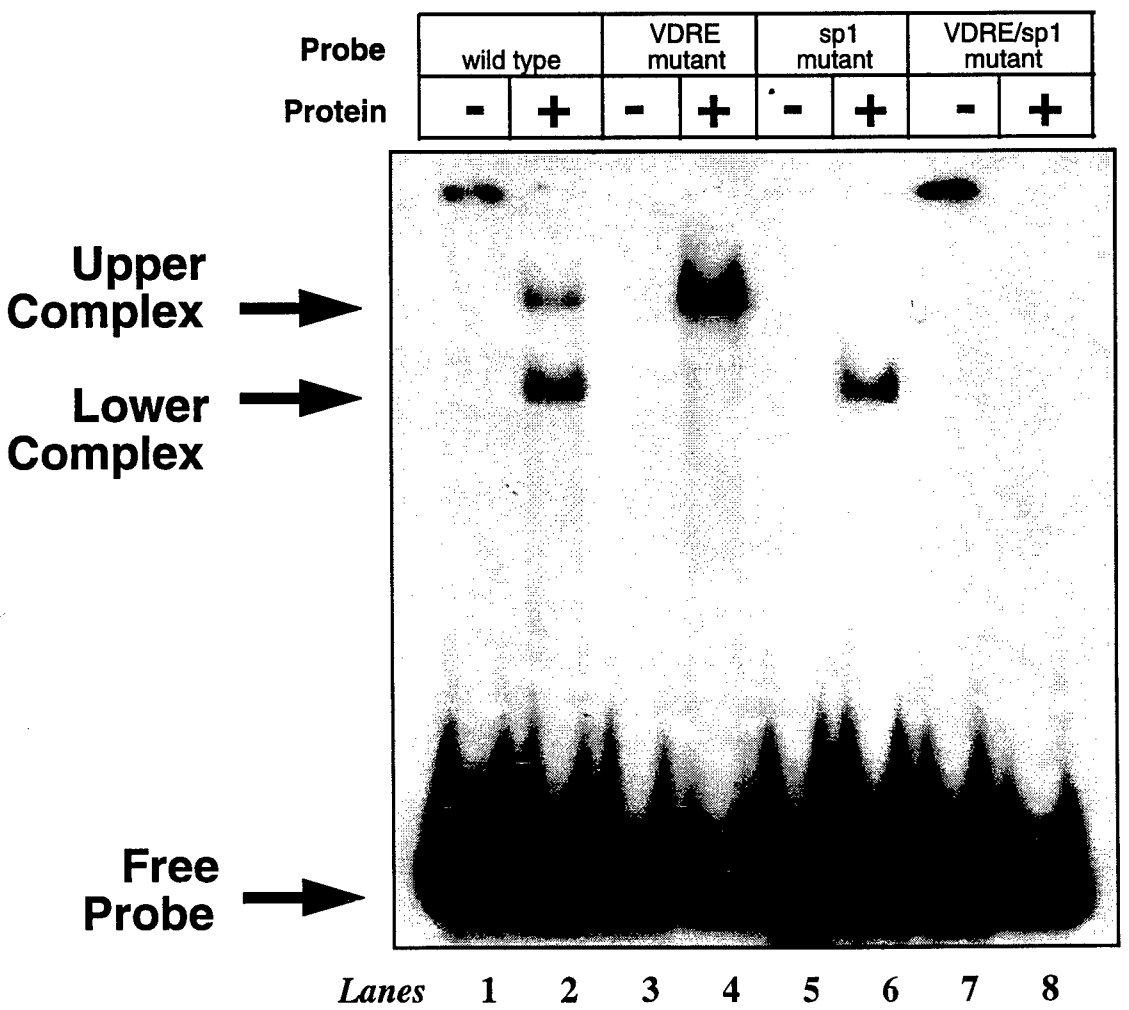


FIGURE 5

FIGURE 6A

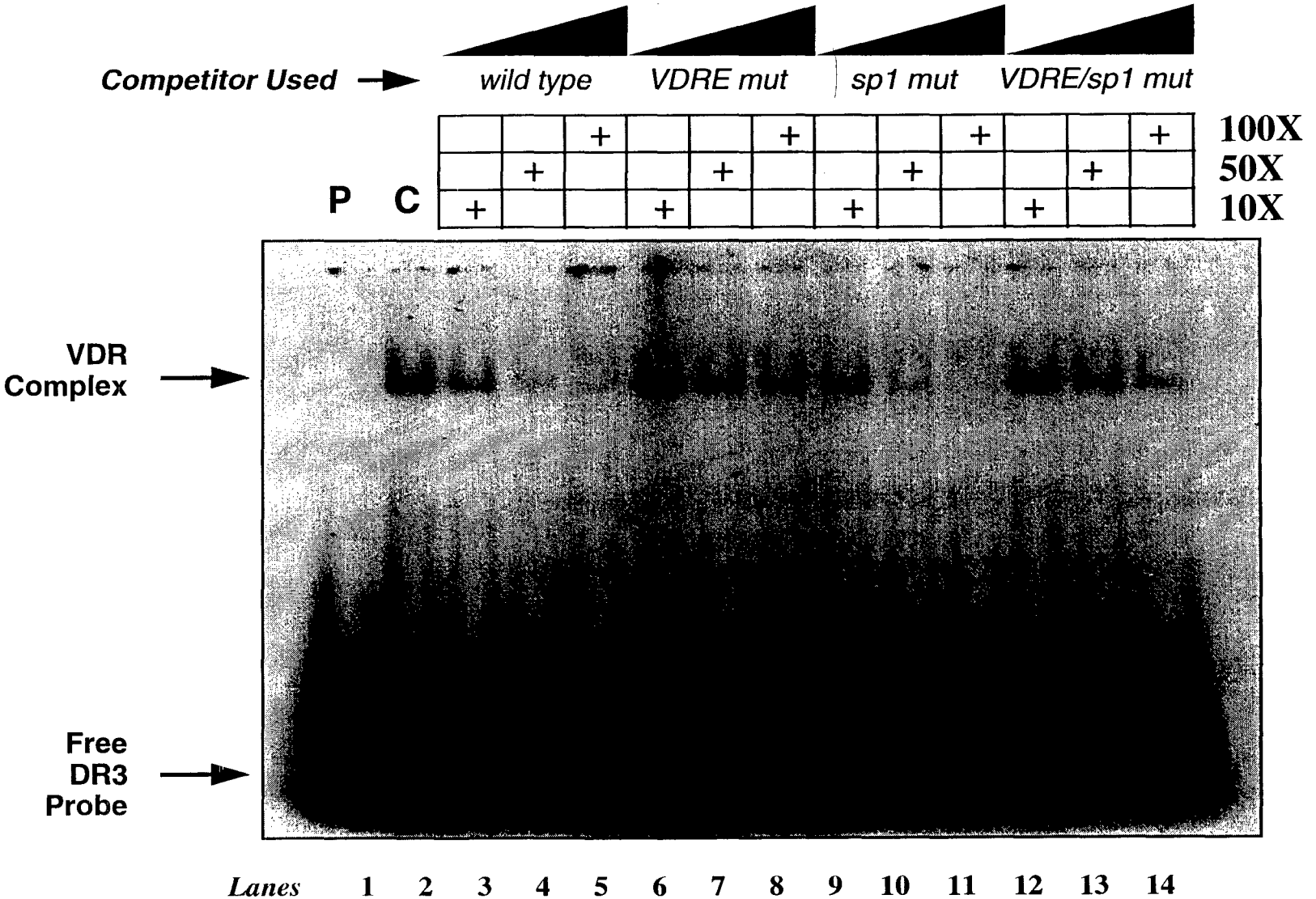

A.
Competitor Used
wild type
VDRE mut
sp1 mut
VDRE/sp1 mut
P
C
100X
50X
10X
VDR Complex
Free DR3 Probe
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 6B

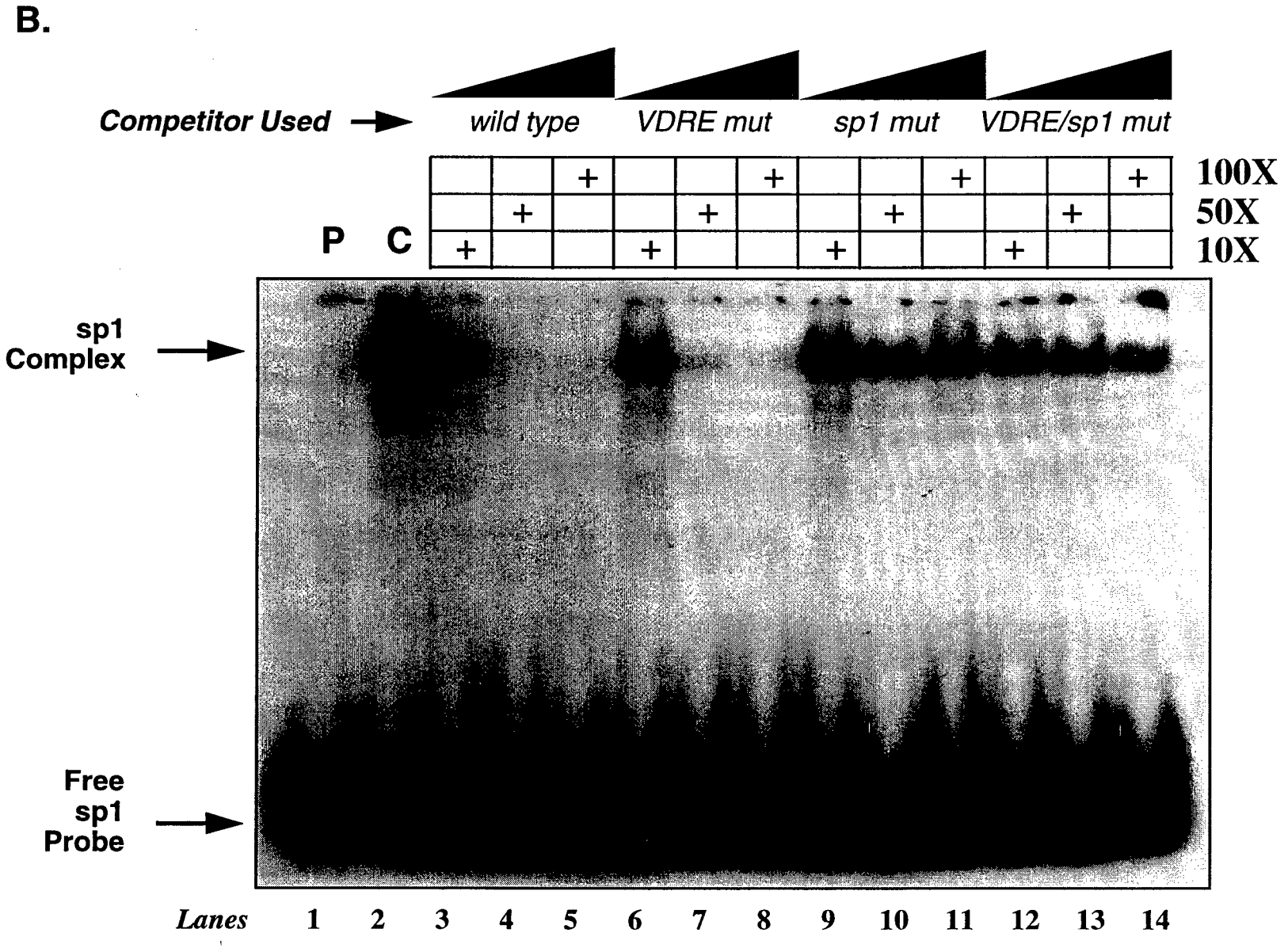

B.
Competitor Used
wild type
VDRE mut
sp1 mut
VDRE/sp1 mut
P
C
100X
50X
10X
sp1 Complex
Free sp1 Probe
Lanes
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 6C

C.

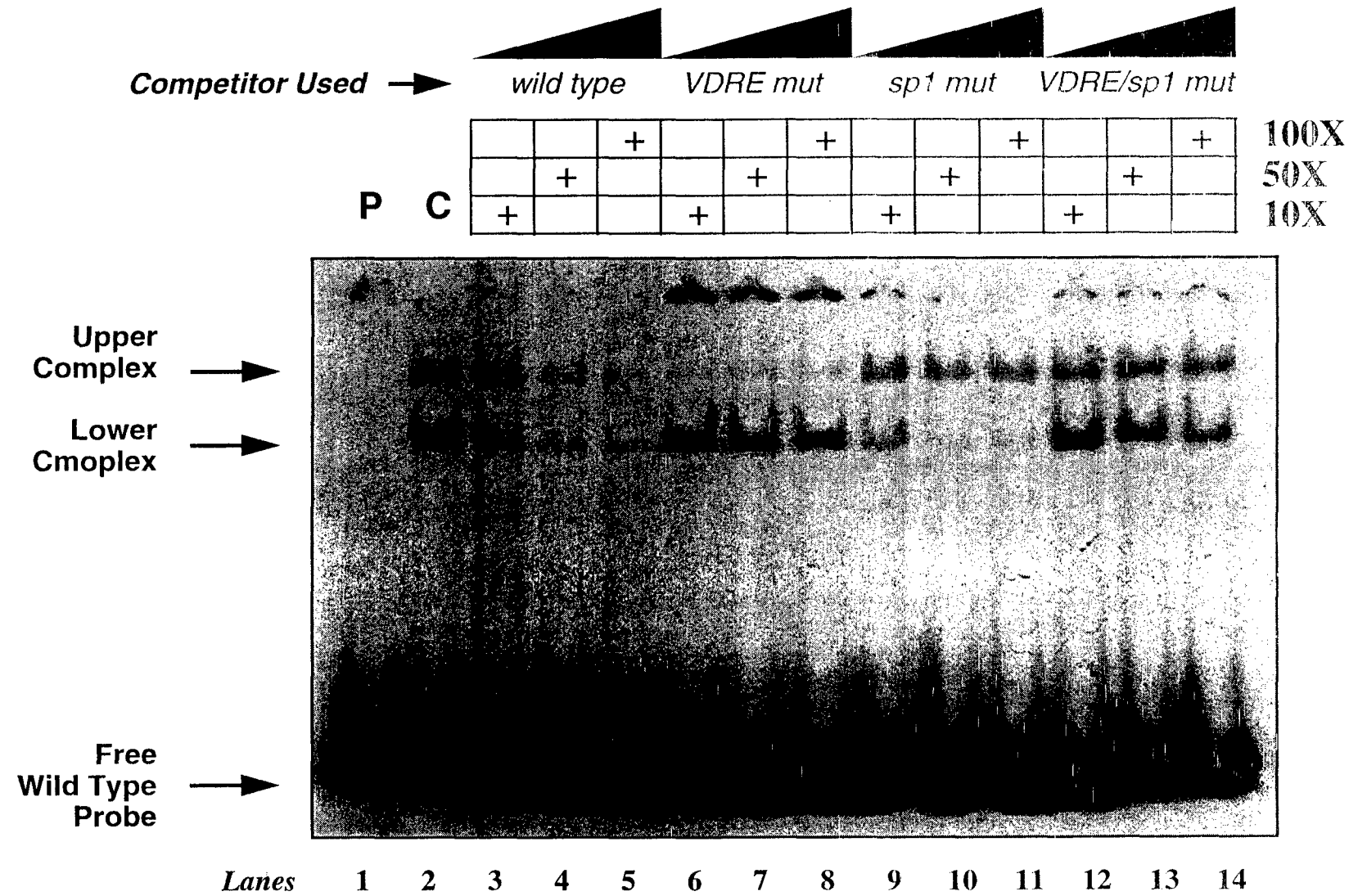
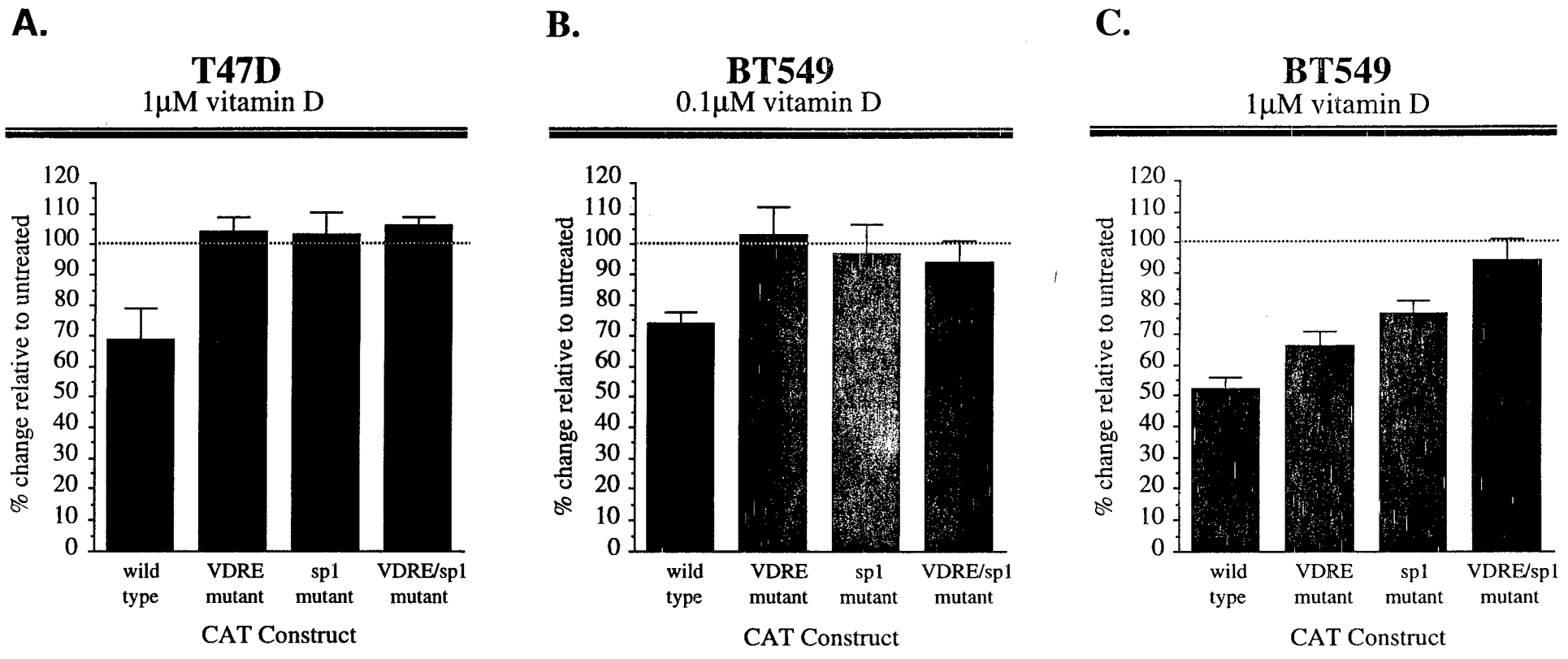

Competitor Used
wild type
VDRE mut
sp1 mut
VDRE/sp1 mut
P
C
100X
50X
10X
Upper Complex
Lower Cmoplex
Free Wild Type Probe
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 7


A.
T47D
1μM vitamin D
% change relative to untreated
120
110
100
90
80
70
60
50
40
30
20
10
0
wild type
VDRE mutant
sp1 mutant
VDRE/sp1 mutant
CAT Construct
B.
BT549
0.1μM vitamin D
% change relative to untreated
120
110
100
90
80
70
60
50
40
30
20
10
0
wild type
VDRE mutant
sp1 mutant
VDRE/sp1 mutant
CAT Construct
C.
BT549
1μM vitamin D
% change relative to untreated
120
110
100
90
80
70
60
50
40
30
20
10
0
wild type
VDRE mutant
sp1 mutant
VDRE/sp1 mutant
CAT Construct

A.

FIGURE 8A

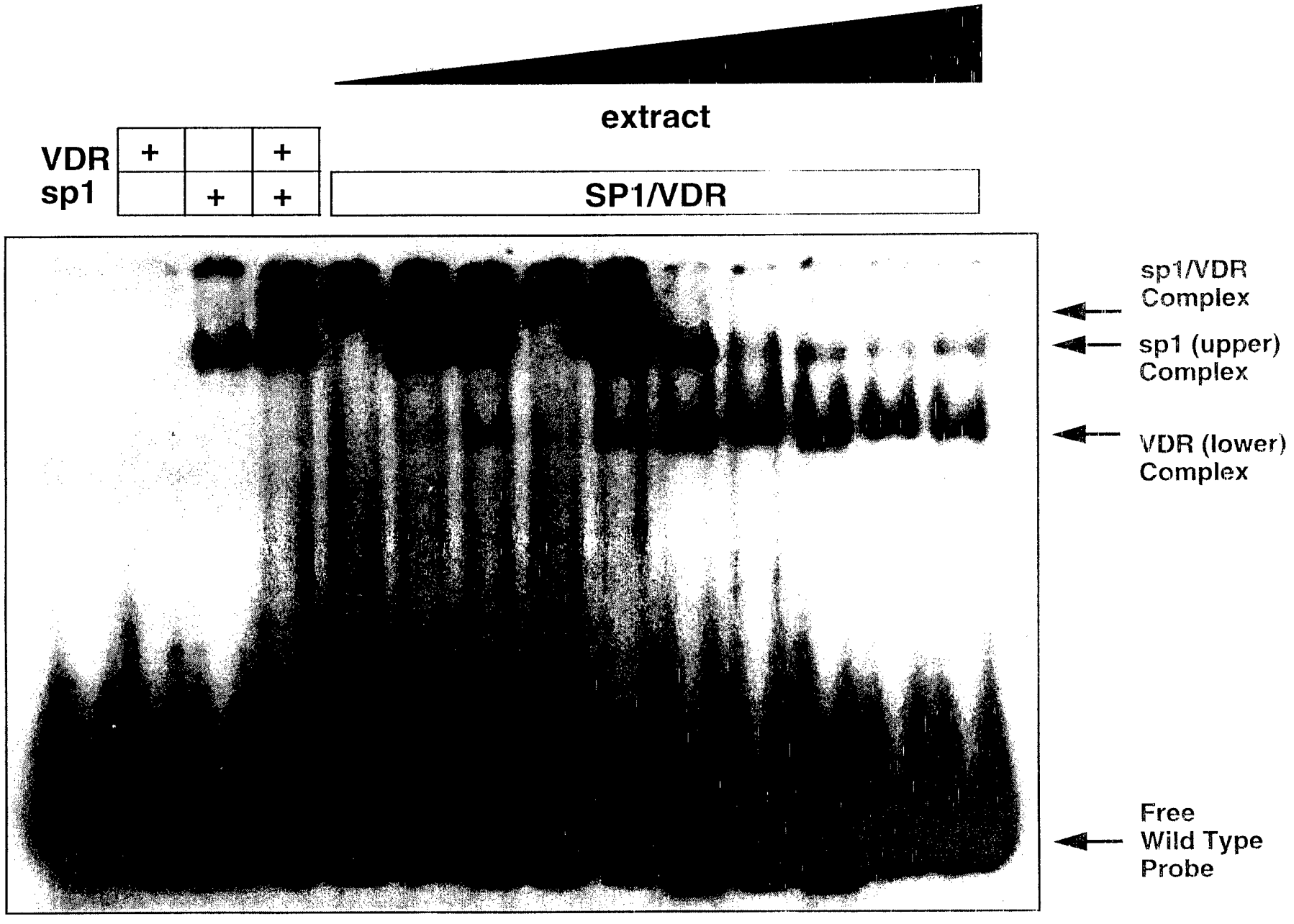
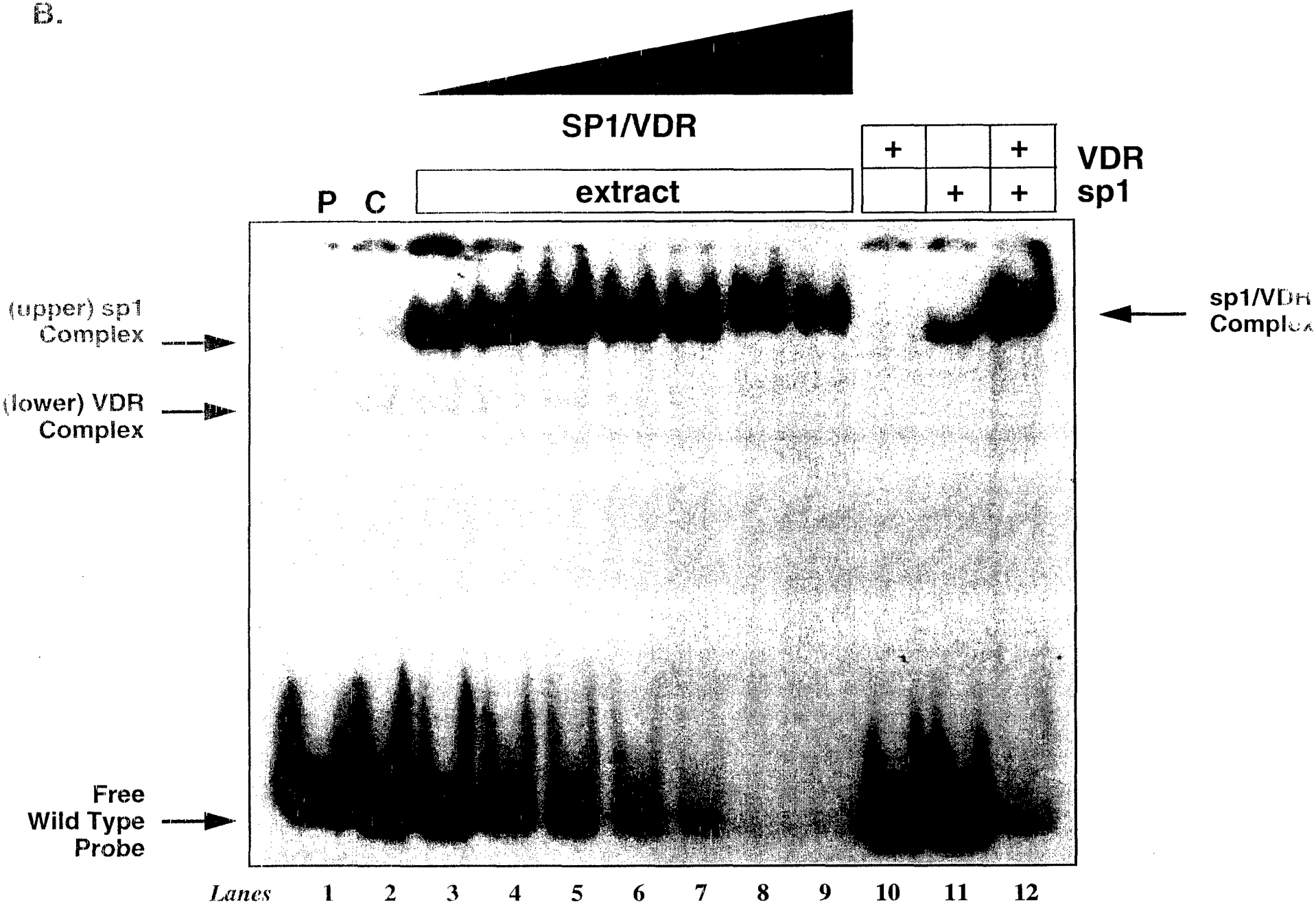

extract
VDR + + +
sp1 + +
SP1/VDR
sp1/VDR Complex
sp1 (upper) Complex
VDR (lower) Complex
Free Wild Type Probe
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 8B

B.


SP1/VDR
+ + VDR
extract + + sp1
P C
(upper) sp1 Complex
(lower) VDR Complex
Free Wild Type Probe
sp1/VDR Complex
Lanes 1 2 3 4 5 6 7 8 9 10 11 12

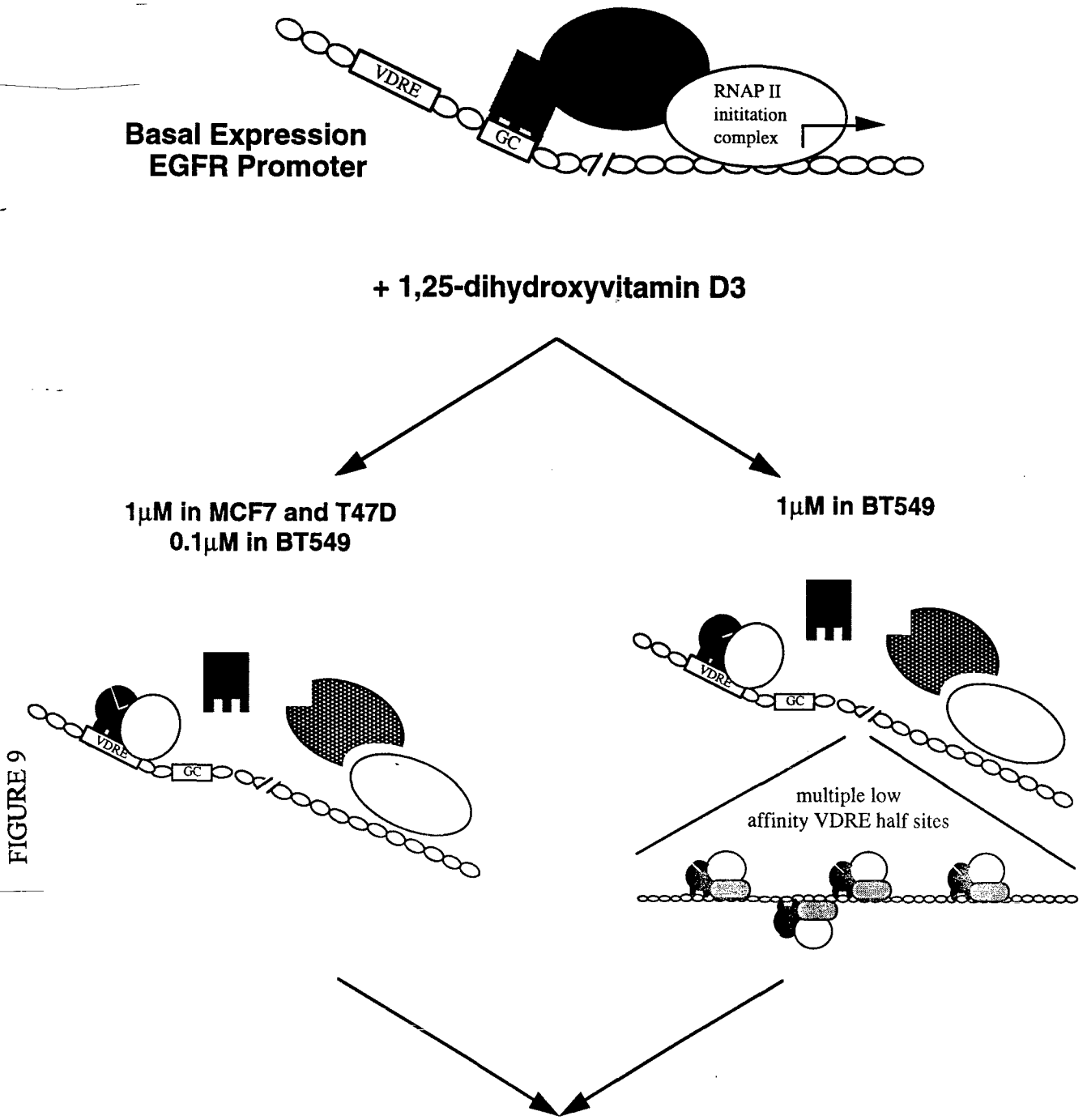


FIGURE 9

= sp1, = coactivator molecule, = RNA polymerase II initiation complex, = 1,25-dihydroxyvitamin D3, = VDR, = unidentified VDR partner, = BT549 cell specific factor

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

DAMD17-94-J-4413	ADB261602
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DAMD17-96-1-6020	ADB244256
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DAMD17-96-1-6098	ADB239338
DAMD17-94-J-4370	ADB235501
DAMD17-94-J-4360	ADB220023
DAMD17-94-J-4317	ADB222726
DAMD17-94-J-4055	ADB220035
DAMD17-94-J-4112	ADB222127
DAMD17-94-J-4391	ADB219964
DAMD17-94-J-4391	ADB233754